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Arenberg Doctoral School
Faculty of Science
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Diversity, community composition and function of arbuscular mycorrhizal fungi in agricultural ecosystems

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Science (Biology)

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September 2016

September 2016

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Uitgegeven in eigen beheer, Maarten Van Geel, september 2016, Leuven

Dankwoord

Eerste en vooral wil ik mijn promotor Olivier bedanken om mij de kans te geven om een doctoraat te voltooien bij zijn onderzoeksgroep. Ik denk niet dat ik mij een betere promotor had kunnen wensen. Je gaf me vrijheid en vertrouwen om de planning en uitwerking van mijn doctoraat zelf te bepalen, je vroeg regelmatig geïnteresseerd naar de stand van zaken en je stond steeds paraat om te antwoorden op vragen. Bovendien verbeterde je manuscripten aan een onmenselijk tempo. Zelfs als je in de Brenne studenten begeleidde, stuurde je me snel een verbeterde versie met de woorden: "Hierbij Maarten. Ik had hier een uurtje vrij." Daarenboven kreeg ik tussendoor nog waardevolle MTB tips en controleerde je regelmatig de bandendruk van mijn fiets (7 bar is perfect!).

Verder wil ik mijn co-promotor Bart bedanken om mij in te leiden in de wereld van de microbiologie en de bijhorende moleculaire methoden. Het was bijzonder aangenaam met je samen te werken en je maakte steeds tijd voor een vergadering of telefoontje, ook al zat je agenda overvol en zag ik je vaak (letterlijk) rennen van de ene afspraak naar de andere. Ik wil ook de collega's van Scientia Terraea, en in het bijzonder Michael Waud, bedanken voor hun hulp bij het labowerk.

Een speciaal woord van dank gaat uit naar Tobias. Dankzij jouw thesisonderwerp kwam ik in contact met onze onderzoeksgroep. Na een succesvolle thesis vloeide hieruit een doctoraat voort en werden we zelfs bureaugenoten. Ik bewonder je vat vol kennis dat steeds overloopt, en eenmaal het aan het overlopen is, nog maar moeilijk te stoppen valt. Hopelijk kunnen veel van je ideeën werkelijkheid worden en vinden ze hun weg naar de wetenschappelijke literatuur. Bedankt voor al je gevraagde (en ongevraagde) suggesties. Naast wetenschappelijke relevante zaken leerde je me ook ABBA en de 'Neue Deutsche Welle' kennen. Hopelijke beleven we volgend jaar nog vele fijne badmintonmomenten.

Graag zou ik ook mijn enthousiaste en immer vrolijke bureaugenoot Matthias willen bedanken. Omdat we samen een bureau deelden, brachten we veel tijd met elkaar door. We leerden elkaar goed kennen, maar er loerde ook een 'Matthias-overdose' om de hoek. Gelukkig trok je elk jaar een paar maand naar de Afrikaanse 'brousse' zodat ik even kon afkicken. Desalniettemin, werden je wetenschappelijke vaardigheden, je enthousiaste discussies, onze praatjes over koetjes en kalfjes, en zelfs je gefluit in de gang tijdens je afwezigheid gemist. Ik wens je veel succes met je verdere carrière en hopelijk kan ik je in de nabije toekomst bezoeken in het verre Zuid-Afrika. Intussen kunnen we af en toe nog eens 'whatsappen', of 'skypen' met giraffen op de achtergrond.

Dank aan Kasper voor zijn hulp bij het veldwerk en het analyseren van de bodemstalen, en Timmy en Gerrit voor hun assistentie bij de DNA analysen. Dank aan alle collega's die steeds mijn vragen beantwoordden, en die zorgden voor de gezellige sfeer op het werk.

Ik wil mijn familie en vrienden bedanken voor hun steun en het voorzien van afleidende momenten. Zonder jullie was het niet gelukt! Hopelijk begrijpen jullie nu beter waar ik vier jaar aan heb gewerkt.

Tenslotte wil ik Tara, mijn veilige haven, bedanken voor haar onvoorwaardelijke steun en om mijn oxytocine niveau op pijl te houden.

Summary

Arbuscular mycorrhizal fungi (AMF) are widespread and obligate plant symbionts, known to play a key role in the functioning of natural and agricultural ecosystems. These fungi establish a symbiosis with the majority of the land plants and generally provide mineral nutrients to the host plant in exchange for plant assimilated carbohydrates. Moreover, they form a large network of hyphae and produce glomalin-related soil proteins that have a great impact on soil formation and soil aggregation. Furthermore, AMF can provide protection of the host plant against abiotic and biotic stress. Given the beneficial effects of AMF to the host plant, their particular value for the sustainable functioning of agricultural ecosystems, and the current environmental threats to AMF diversity, it is crucial to identify the major environmental drivers of AMF communities in agricultural settings.

In this PhD dissertation, we presented research on diversity, community composition and function of arbuscular mycorrhizal fungi AMF in agricultural ecosystems. The general aim of this research was to gain a better understanding of how environmental factors, such as site geography, soil properties and management, influence the diversity and community composition of AMF in agricultural systems. Additionally, we aimed to assess the role of AMF specificity and diversity on crop performance. In this research, we performed a meta-analysis, two observational studies, a field experiment and compared methods to characterize AMF communities.

In chapter two, we evaluated six primer pairs targeting the nuclear rRNA operon for characterization of AMF communities using 454 pyrosequencing. We showed that primer pairs AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 outperformed the other tested primer pairs in terms of number of Glomeromycota reads (AMF specificity and coverage). Additionally, these primer pairs were found to have no or only few mismatches to AMF sequences and were able to consistently describe AMF communities from apple roots. However, whereas most high-quality AMF sequences were obtained for AMV4.5NF-AMDGR, our results also suggest that this primer pair favored amplification of Glomeraceae sequences at the expense of Ambisporaceae, Claroideoglomeraceae and Paraglomeraceae sequences.

In chapter three, we studied the response of AMF in the roots of cultivated apple trees with increasing orchard fertilization. We characterized the diversity present in the apple roots using 454-pyrosequencing and investigated which environmental factors drive AMF diversity and community composition. We showed that soil characteristics and management type, rather than the geographical location of the orchards, shape AMF communities in the roots of apple trees. Particularly, plant-available P content of the soil was negatively correlated to AMF diversity. Finally, the degree of nestedness of the AMF communities was related to plant-available P and N content of the soil, indicating at a progressive loss of AMF taxa with increasing fertilization.

In chapter four, we experimentally tested whether different amounts and forms of phosphorus fertilizer affected AMF diversity and community composition associated with the roots of apple trees. We found that the slow-release fertilizer treatments showed significantly higher AMF

richness and differed in community composition compared to the inorganic fertilizer treatments. The distribution of AMF OTUs showed a significantly nested pattern. Additionally, AMF communities in the inorganic fertilizer treatments were a subset of the communities in the slow-release fertilizer treatments.

In chapter five, we focused on the AMF diversity present in vine roots and investigated which environmental factors drive AMF diversity and community composition. We found no differences in AMF diversity between organically and conventionally managed vineyard. Instead, plant-available P content of the soil and pH, which were similar in both organically and conventionally managed vineyards, were the only variables significantly related to AMF diversity. In agreement with our diversity analysis, we found that available P content in the soil was also significantly related to AMF community composition. The effect of management type on AMF community composition, however, was unclear, indicating management type could explain very little variation in AMF community composition. Although we found no effects of copper concentration in the soil on AMF diversity and community composition, we observed that older vineyards (> 15 years) showed copper concentrations above the background level (30 mg/kg). Additionally, we found that the AMF communities occurring in the roots of grapevine were organized in a nested pattern. The degree of nestedness was positively correlated to the plant-available P in the soil and negatively correlated to soil acidity.

In chapter six, we applied meta-analytical techniques on the literature reporting on the response of crop plants to examine the effect of crop identity, AMF identity, and AMF diversity on crop biomass increase, following inoculation. Our results showed that total crop biomass was on average 34.9% higher in inoculated versus non inoculated plants. We found that specific combinations of AMF genera and host plant families were more beneficial for growth promotion as compared to other combinations. Moreover, a single-species inoculum increased crop growth response on average by 41.2% compared to a multi-species inoculum.

In both our observational studies (chapter 3 and 5) and our field experiment (chapter 4), the available P in the soil was the most important factor that influenced AMF diversity and community composition. Likewise, we also found distinct AMF communities in orchards and vineyards with a high and low plant-available P content of the soil (chapter 3 and 5). Next to the available P in the soil, AMF diversity was also related to soil acidity in vineyards (chapter 5), but not in apple orchards (chapter 3). This can be explained by the larger sample size in chapter five, which increased the statistical power and thus the ability to detect the smaller effect of pH on AMF diversity. Moreover, our results unambiguously indicate that enrichment of P levels in the soil through fertilization results in a gradual loss of AMF specialists and communities dominated by generalists, such as *Rhizophagus intraradices*, which may be inferior mutualists. The effects of organic management on AMF diversity were inconclusive. In apple orchards, organic management increased AMF diversity (chapter 3). We found, however, no differences in AMF diversity between conventionally and organically managed vineyards (chapter 5). The main difference between these studies is that the organically managed apple orchards all had low available P levels (< 100 mg P/kg), while available P

levels in the soil of organically managed vineyards ranged considerably and did not differ from conventionally managed orchards. On the one hand current available P levels in the soil may be explained by the time since conversion to organic farming and the prehistory of the soil. On the other hand, organic farming is no guarantee for low available P levels, as organic farming allows organic fertilization, which may still elevate P levels in the soil above optimum levels. Therefore, we recommend organic management in combination with low to moderate fertilization using slow-release fertilizers to preserve diverse AMF communities in agricultural ecosystems.

In chapter six, we showed that crop biomass increased on average 34.9% in inoculated plants compared to non-inoculated plants, and that a multi-species inoculum decreased the growth response compared to a single species inoculum. Yet, we were unable to measure the effect of changing AMF diversity and community composition as a result of different soil characteristics and management (chapter 3-5) on crop performance of apple trees and grapevines in the field. Future research should focus on how much complex AMF communities that change with soil conditions and management, contribute to crop performance in the field.

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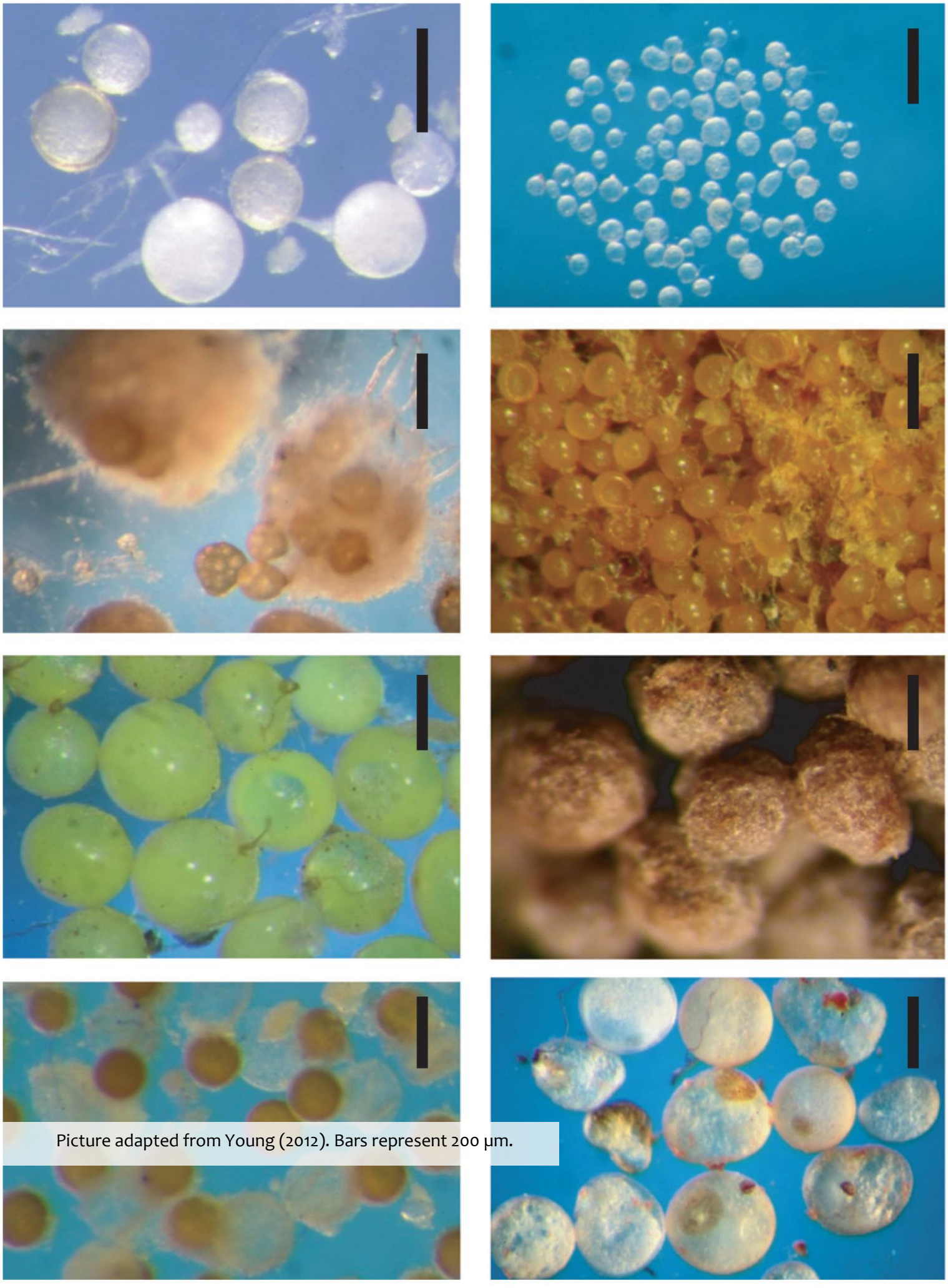
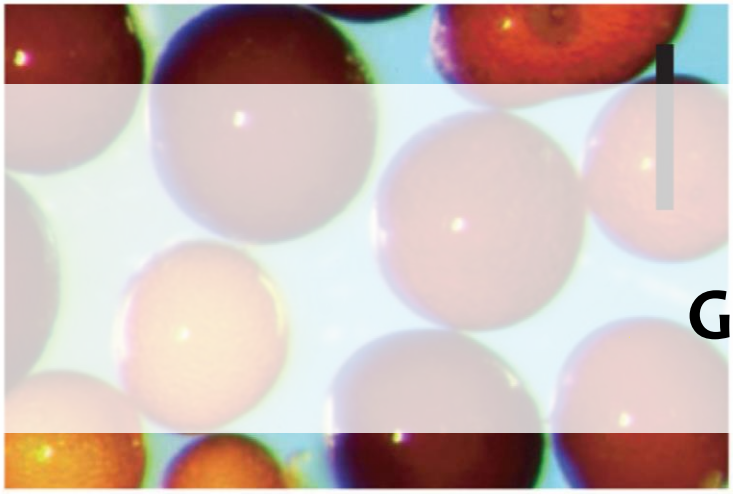
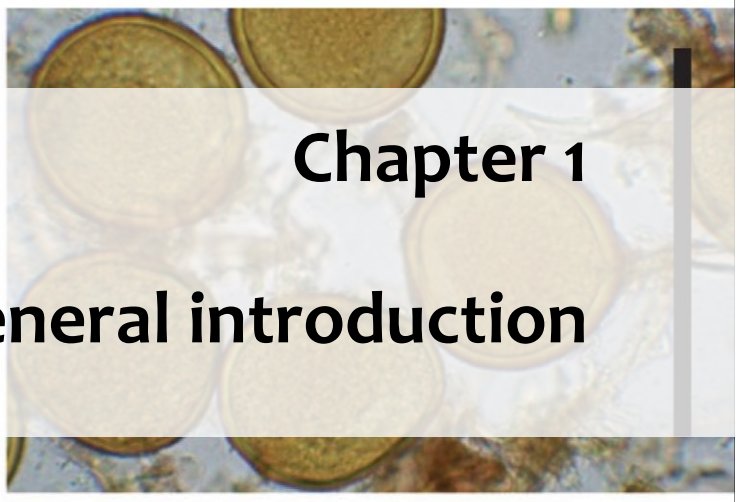
List of abbreviations

AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
BIC	Bayesian Information Criterion
BLAST	Basic local Alignment Search Tool
CO	Control Treatment
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded Deoxyribonucleic acid
Exp(H)	Shannon Diversity Index Exponentially Transposed
GC	Green Compost Treatment
GLM	General Linear Models
IF20	Inorganic Fertilizer 20 treatment
IF50	Inorganic Fertilizer 50 treatment
ITS	Internal Transcribed Spacer Region
LSU	Large Subunit
MC	Mushroom Compost Treatment
N	Nitrogen
NGS	Next Generation Sequencing
NMDS	Non-metric Multidimensional Scaling
OTU	Operational Taxonomic Unit
P	Phosphorus
PCNM	Principal coordinates of neighbor matrices
RDA	Canonical Redundancy Analysis
rRNA	ribosomal Ribonucleic Acid
SSU	Small Subunit
ST	Struvite treatment
T-RFLP	Terminal Restriction Length Fragment Polymorphism

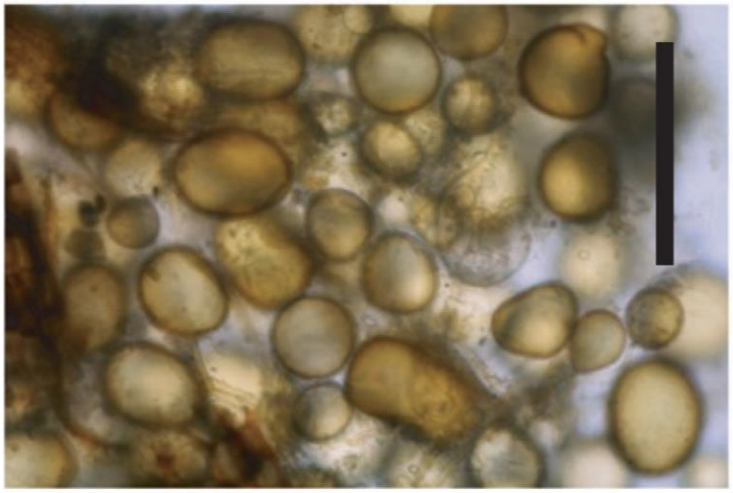


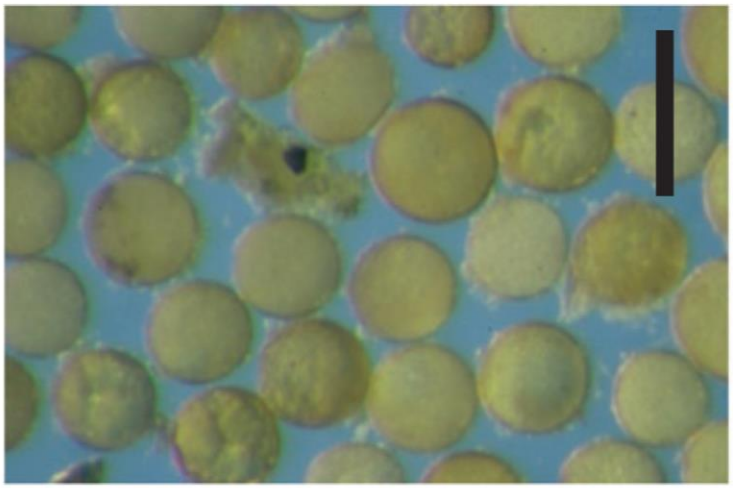
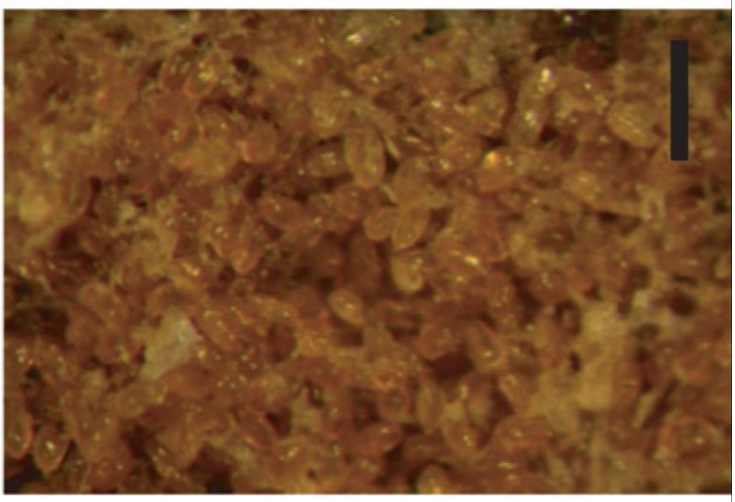
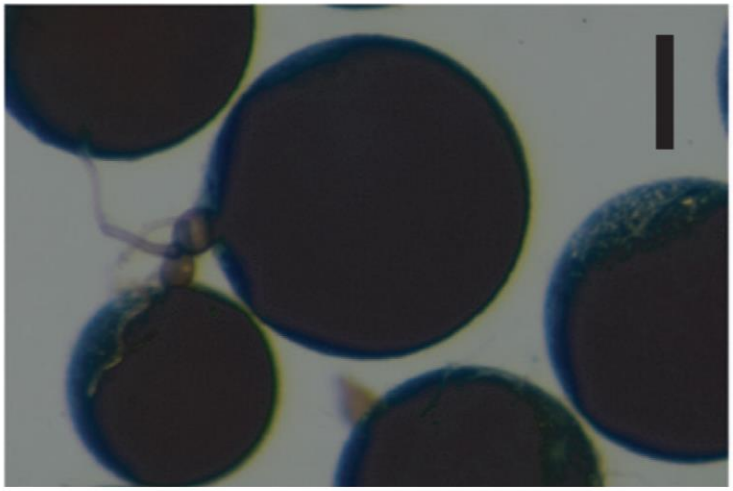
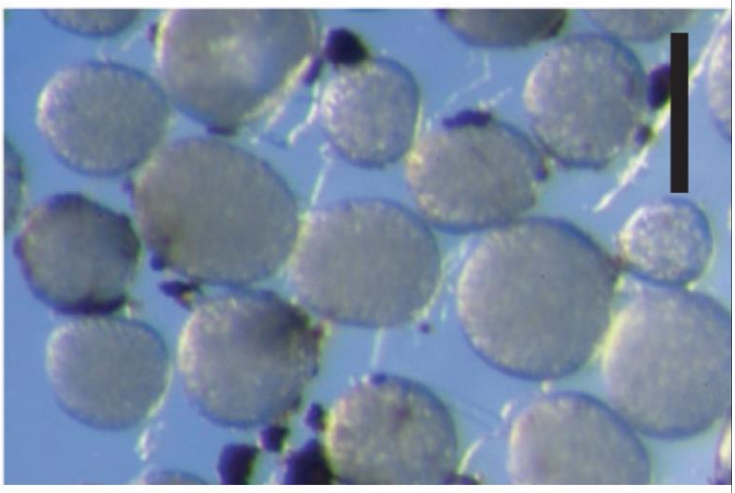
Picture adapted from Young (2012). Bars represent 200 µm.

Chapter 1

General introduction



1.1 The mycorrhizal symbiosis

Relationships among species are often defined by the effect of the interaction on the fitness of each of the interacting species. There is a continuum of interactions ranging from mutually detrimental to mutually beneficial relationships (Begon *et al.*, 2006). For example, a competitive interaction is one in which both organisms are negatively affected. Parasitism occurs when one species gains and the other looses. Finally, a mutualistic relationship is one in which organisms of different species interact to their mutual benefit.

The term 'symbiosis' (meaning living together) was first used by Frank (1877) and implied the regular coexistence of dissimilar organisms. Here, the 'symbiont' occupies a habitat provided by a 'host'. Parasites are usually excluded from the category of symbionts. Instead, the term is usually reserved for the mutualistic symbioses. Mutualistic symbioses are those in which both coexisting partners interact to their mutual benefit (Boucher, 1988).

The mycorrhizal symbiosis is one of the most widespread on earth. The term 'mycorrhiza' (from the Greek 'mycos, meaning fungus and 'rhiza', meaning root) can be defined as the association between the roots of plants and symbiotic soil fungi. Mycorrhizas are often considered as classical mutualisms, although it has been shown they can function along a mutualism-parasitism continuum (Johnson *et al.*, 1997).

1.1.1 Different types of mycorrhizal fungi

The mycorrhizal symbiosis can generally be divided in five major types: the ectomycorrhiza, the arbuscular mycorrhiza (AMF), the orchid mycorrhiza, the ericoid mycorrhiza and the ectendomycorrhiza fungi (Fig. 1.1).

Ectomycorrhizas

Ectomycorrhizal fungi are characterized by the presence of three structural components: the sheath, the Hartig net and the extraradical mycelium (Fig. 1.1). The sheath is surrounding the surface of the root of the host and is formed by a dense hyphal mantle. Unlike other mycorrhizal types, such as arbuscular mycorrhiza and ericoid mycorrhiza, ectomycorrhizal fungi do not penetrate the cells of the root. Instead, they grow between the epidermal and cortical cells of the root. This labyrinth of hyphae inside the root is called the Hartig net. Finally, an outwardly growing system of hyphal elements, i.e. the extraradical mycelium, forms the connection between the soil and the ectomycorrhizal fungi.

Ectomycorrhizal fungi are widespread but associate with only 3% of the vascular plant families, mainly trees and shrubs (Smith & Read, 2008). They play an important role in seedling establishment and tree growth in habitats across the globe (Tedersoo *et al.*, 2010b).

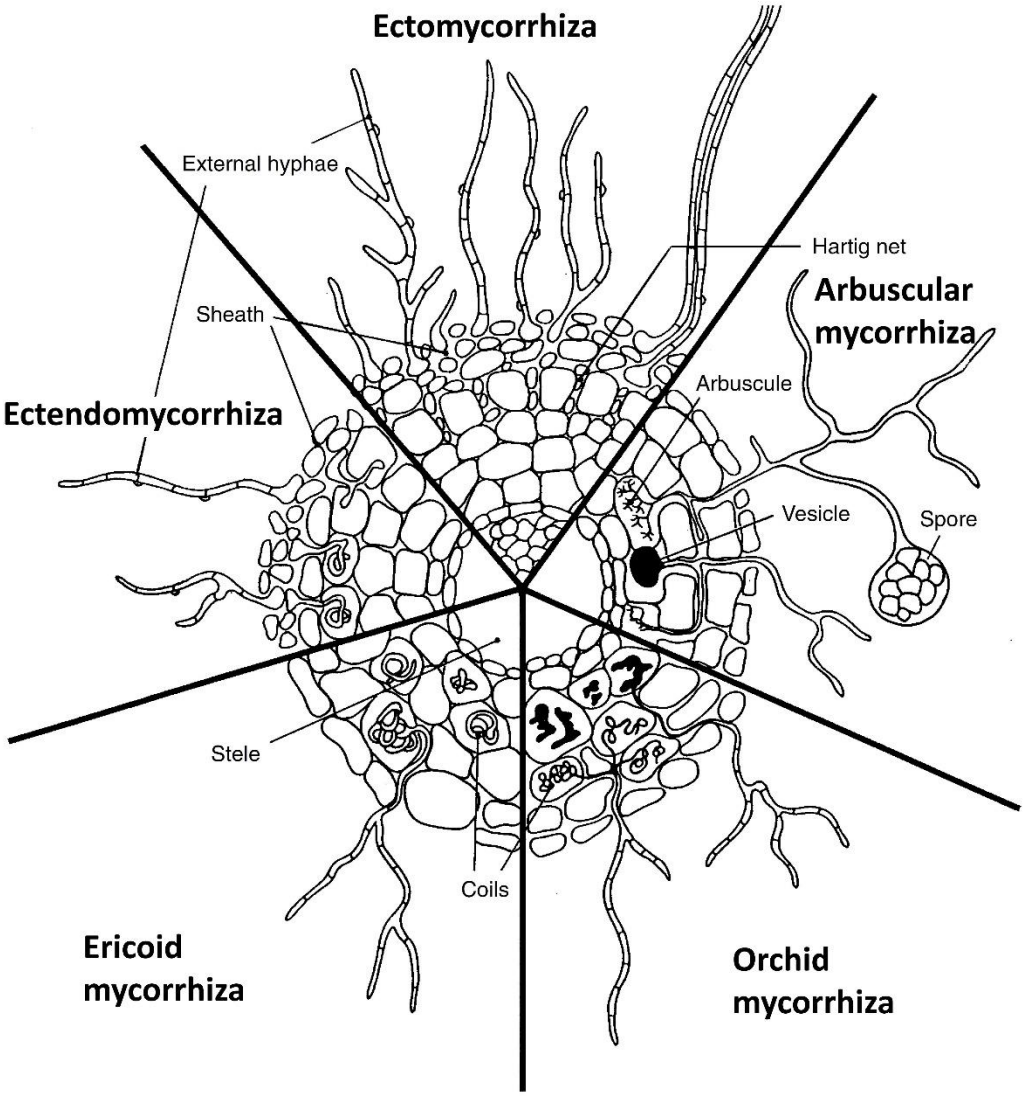


Figure 1.1 A schematic representation showing the structures of the main mycorrhizal types. Adapted from Selosse and Tacon (1998).

Arbuscular mycorrhizal fungi

The arbuscular mycorrhizal association with roots is probably the most ubiquitous terrestrial symbiosis. Therefore, the arbuscular mycorrhizal symbiosis is often called the mother of plant root endosymbiosis (Parniske, 2008). AMF, which belong to the monophyletic phylum Glomeromycota (Schüssler *et al.*, 2001), form a symbiosis with 70 – 80% of all land plant species (Smith & Read, 2008). In contrast to the Ectomycorrhiza, the development of AMF results in the formation of tree-shaped subcellular structures within the cells of the plant root. These structures are known as arbuscules (from the Latin 'arbusculum', meaning bush or little tree) and are thought to be the main site of nutrient exchange between both symbiotic partners (Parniske, 2008; Fig. 1.1). The extraradical hyphal network, which can exceed 100 meters of hyphae per cubic centimeter of soil (Miller *et al.*, 1995), acts as a living interface between plant roots and the soil. This hyphal network will provide a range of benefits to the host plant (which we will discuss in depth below), including improved

nutrient and water uptake. In exchange, AMF acquire carbohydrates from the plant (Solaiman & Saito, 1997).

Orchid mycorrhiza

The Orchid and Ericoid mycorrhiza are, just like the arbuscular mycorrhiza, endomycorrhiza, which means that the fungus also enters the inside of the roots cells (Fig. 1.1). Orchid mycorrhiza are mainly fungi from the phylum Basidiomycota that form a symbiosis with the roots of plants of the family Orchidaceae (Rasmussen, 2002). They are specifically important for orchid seed germination and establishment, because orchid seeds contain virtually no energy reserve, making them highly dependent on sugars provided by the fungus (McCormick *et al.*, 2012).

Ericoid mycorrhiza

Ericoid mycorrhiza are fungi from the phylum Ascomycota that form a symbioses with the uniquely specialized distal roots of plants of the Ericaceae, which predominantly occur in heathland ecosystems. Ericoid mycorrhiza are adapted to harsh, acidic and nutrient poor conditions that species of the Ericaceae typically inhabit (Cairney & Meharg, 2003). They are characterized by fungal coils that form in the epidermal cells of the fine hair roots of their host (Fig 1.1). Here, they exchange nutrients obtained from the soil for carbohydrates synthesized by the plant.

Ectendomycorrhiza

Ectendomycorrhiza demonstrate characteristics of both the ectomycorrhiza and endomycorrhiza, i.e. they show extensive intracellular penetration, the Hartig net and simultaneously the presence of a sheath of hyphae surrounding the root of the plant (Fig. 1.1). They primarily occur in *Pinus* and *Larix* species (Yu *et al.*, 2001).

In this thesis, we will focus on the arbuscular mycorrhizal fungi.

1.2 The arbuscular mycorrhizal symbiosis

AMF are thought to exist ever since plants colonized the land surface, at least 400 million years ago (Ordovician) (Redecker *et al.*, 2000). They are obligate biotrophs, meaning they are not capable of making their own carbon and depend solely on the photosynthetic products of their host plant to complete their life cycle and produce the next generation of spores. It is estimated that AMF can consume up to 20% of the host's photosynthate for establishment and maintenance (Bago *et al.*, 2003).

AMF are asexual organisms that use their spores for dispersal to new habitats or to initiate a new spatially separated individual (Morton *et al.*, 1993). Their hyphal network is aseptate (without septa) and coenocytic, meaning hundreds of nuclei can share the same cytoplasma. Likewise, individual spores can also contain multiple nuclei. Although these organisms are thought to be asexual, genetic material can be exchanged and recombined through anastomosis between genetically different AMF (Croll *et al.*, 2009). Therefore, anastomosis allows many genetically different individuals of a population to connect and form a large genetically diverse hyphal network. Additionally, by random distribution of nuclei at spore formation, the progeny of an AMF

could receive different complements of nucleotypes compared to their parents or siblings (Angelard *et al.*, 2010). Therefore, segregation and genetic exchange through anastomosis may be two mechanisms that can create new progeny with different symbiotic effects in a very short time span and allow AMF to adapt rapidly to different environmental conditions (Angelard & Sanders, 2011).

1.2.1 Classification and phylogeny

In 2001, the AMF were separated in a new phylum: the Glomeromycota (Schüssler *et al.*, 2001). The Glomeromycota are closely related to the Basidiomycota and the Ascomycota. Although considerable confusion existed in the taxonomy of the Glomeromycota, an evidence-based consensus classification based on both morphological and DNA sequence data was only published in 2013 (Redecker *et al.*, 2013). The Glomeromycota are divided in four orders (the Glomerales, Diversisporales, Archaeosporales and Paraglomerales) (Fig. 1.2), because available sequence data indicate they are near-equivalent sister groups (Schüssler *et al.*, 2001; Redecker *et al.*, 2013). These orders are further subdivided in 10 families and 19 genera that are supported by sufficient evidence (Fig. 1.2).

Currently, there are 265 morphospecies of AMF described (A. Schüssler's Glomeromycota phylogeny, http://schuessler.userweb.mwn.de/amphylo/; 12 January 2016). However, the use of SSU rRNA sequence groupings (called virtual taxa, VT) as a proxy for the species level allowed natural occurrence patterns of AMF to be described at the DNA level (Öpik *et al.*, 2013). The Maarjam database, an online curated database for the Glomeromycota (Öpik *et al.*, 2010), now comprises of 352 virtual taxa (http://maarjam.botany.ut.ee/, 12 January 2016). These virtual taxa include 71 of the morphospecies already described and many sequence groupings that may present yet undescribed species of Glomeromycota (Öpik *et al.*, 2013). Additionally, Kivlin *et al.* (2011) suggest that the global AMF richness is much higher than previously thought (563 SSU rRNA sequence groupings), largely due to a high beta diversity among sampling sites.

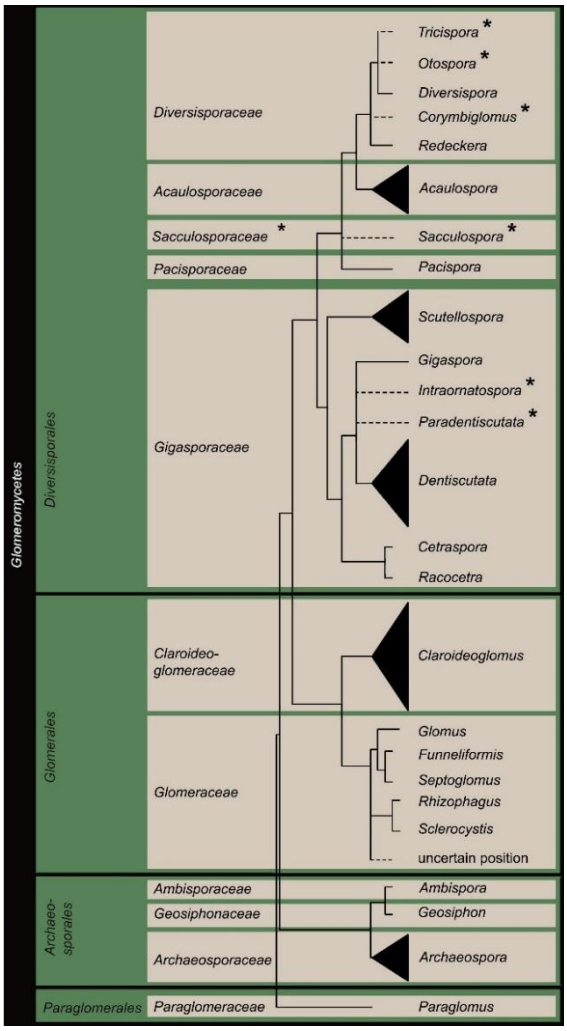


Figure 1.2 In 2013, Redecker *et al.* (2013) proposed an evidence-based consensus classification for the arbuscular mycorrhizal fungi (Glomeromycota). Asteriks indicate insufficient evidence. Adapted from Redecker *et al.* (2013).

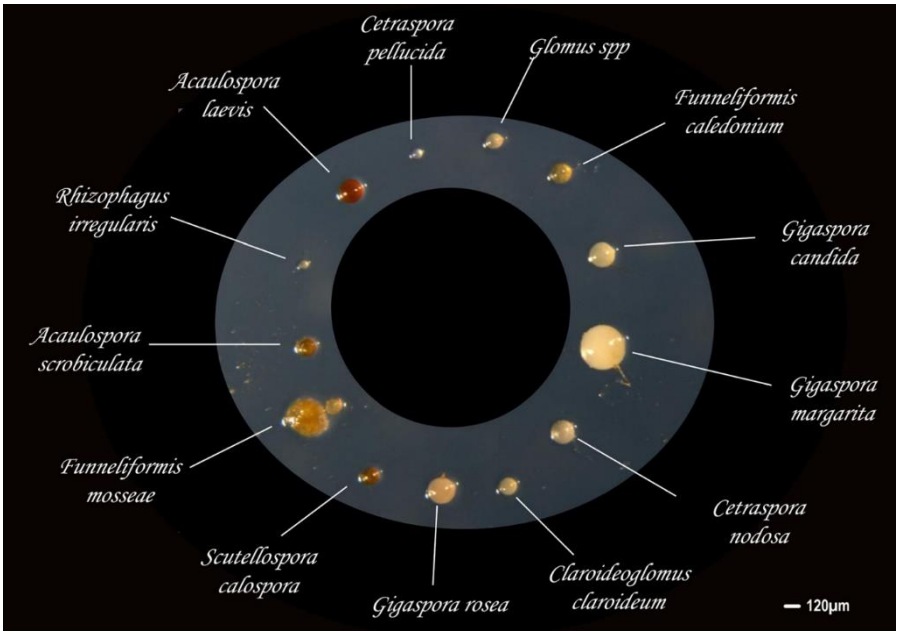


Figure 1.3 Spores from different species of arbuscular mycorrhizal fungi. Adapted from Trouvelot *et al.* (2015).

1.2.2 Identification of arbuscular mycorrhizal fungi

The methods for identifying and analysing AMF communities have undergone a profound change in the last two decades. Traditionally, AMF communities were characterized using microscopic analysis of the spores (Fig. 1.3) (Gorzelak *et al.*, 2012). This approach may be flawed because of the limited morphological differentiation of the spores. Some families share, for example, the same morphological characters, while other species can potentially form dimorphic spores (Morton & Redecker, 2001). In addition, sporulation is very seasonal and some AMF taxa even do not sporulate at all (Smith & Read, 2008). In the absence of spores, the microscopic analysis based on hyphal structures allows identification to the family level at best (Merryweather & Fitter, 1998). Additionally, several AMF taxa do not stain, or stain weakly, using standard dyes, making identification with microscopic analysis impossible (Gorzelak *et al.*, 2012). Finally, the identification of AMF using microscopic techniques is prone to human errors which may lead to misidentification. Therefore, it is very likely that researchers in the past underestimated AMF diversity and misrepresented the true composition of AMF communities.

Molecular approaches have become the standard approach for characterizing AMF communities. By this means the need for microscopic analysis is circumvented. There are many molecular techniques to characterize the AMF community present in an environmental sample. Basically, they can be divided in community profiling and sequencing techniques.

Community profiling techniques

Community profiling techniques, also called genetic fingerprinting, use base pair differences to separate sequences from different AMF taxa to create a unique genetic fingerprint for each composite sample. These base pair differences are, for example, identified through subjecting the sequences to a chemical or temperature denaturant (Denaturing Gradient Gel Electrophoresis), or by differences in sequence length (Terminal Restriction Length Fragment Polymorphism).

Denaturing Gradient Gel Electrophoresis (DGGE) uses a chemical gradient to denature the sample as it moves across an acrylamide gel. Temperature Gradient Gel Electrophoresis (TGGE) is a refinement of DGGE. Instead of a chemical gradient, it uses a temperature gradient to denature the sample. Base pair differences in fragments of the same length will cause them to denature at different positions in the gel. When the sequences denature, their movement will halt at a precise point within the gel that is unique to each sequence. Therefore, this technique allows to generate a presence/absence data matrix for each unique sequence and for each sample.

Terminal Restriction Length Fragment Polymorphism (T-RFLP), another profiling technique, was first described by Liu *et al.* (1997) and is based on digesting fluorescently labelled amplicons with one or more restriction enzymes. Subsequently, the mixture of fragments are separated using polyacrylamide electrophoresis and the size of the labelled terminal fragments is determined. Because the restriction enzymes cuts the sequences at a specific base pair sequence, known as the restriction site, different sequences can have different restriction sites. Therefore, a unique profile for each composite sample will be generated depending on the composition of the sample.

However, some taxa may produce identical banding patterns, which results in an underestimation of the richness (Dickie & FitzJohn, 2007). Especially, closely related AMF species may not be easily resolved as they share the same restriction sites (Gorzelak *et al.*, 2012). Nevertheless, many studies utilized T-RFLP to characterize the AMF community, f.e. Vandenkoornhuyse *et al.* (2003) and Verbruggen *et al.* (2010).

Following the restriction reaction, the mixture of fragments is separated using either capillary or polyacrylamide electrophoresis in a DNA sequencer and the sizes of the different terminal fragments are determined by the fluorescence detector. Because the excised mixture of amplicons is analyzed in a sequencer, only the terminal fragments (i.e. the labeled end or ends of the amplicon) are read while all other fragments are ignored. Thus, T-RFLP is different from ARDRA and RFLP in which all restriction fragments are visualized.

Although community profiling techniques are affordable and fast, they do not provide taxonomic information about the different AMF present in the sample. For this, you need sequencing techniques.

Sequencing techniques

Based on Sanger sequencing, only one piece of DNA can be sequenced at a time. Therefore, the sequences from the amplicon are separated and cloned into competent cells (cells that are able to take up exogenous genetic material) that can be individually sequenced. Sanger sequencing is based on the selective incorporation of labeled chain-terminating nucleotides by polymerase. The labeled terminal nucleotides are read using an automatic sequencer, allowing to reconstruct the original sequence (Sanger, 1977). Although Sanger sequencing is still used for research requiring a low number of sequences per sample (e.g. Alguacil *et al.*, 2011) or to validate next-generation sequencing methods (e.g. Tedersoo *et al.*, 2010a), it has a low yield of sequences per sample and relative high cost per sequence.

High-through sequencing, or next-generation sequencing (NGS), refers to various technologies that perform parallel sequencing, allowing to sequence multiple pieces of DNA or RNA at once. Therefore, besides other purposes, NGS is ideal to sequence amplicons from environmental samples and have the potential to dramatically accelerate ecological and environmental research. In comparison to classic Sanger sequencing, NGS produces an enormous amount of sequencing information about the microbial communities (Schuster, 2008), making it the first choice for many areas of microbial research. In 2009, the first studies appeared that used NGS technologies for the identification of AMF (f.e. Öpik *et al.*, 2009; Lumini *et al.*, 2010). They utilized the first NGS commercially available platform: the FLX Genome Sequencer marketed by Roche Applied Science (454), known as 454 pyrosequencing. This system produces over 1 million sequences per run and was preferred over other NGS platforms as it allowed sequences up to 450 bp (and later 800 bp with the FLX+) (Rothberg & Leamon, 2008). Illumina sequencing, another manufacturer of NGS platforms, is currently the most successful and most widely adopted technology in biological sciences. However, it has not been adopted for analysis of fungal communities due to the limited

read length (250 bp). According to the manufacturer, it is now possible to produce 2 x 250 bp pair-ended sequences and a yield of 30 million reads with the Illumina miseq (Lindahl *et al.*, 2013).

Whichever sequencing technology is used, sequencing-based characterization of microbial communities generally relies on the amplification (with PCR) of phylogenetically relevant markers using general primers, followed by sequencing. For AMF, the nuclear ribosomal RNA (rRNA) operon is commonly used, mainly due to its high resolving power and alignment capability across broad taxonomic groups (Stockinger *et al.*, 2010; Schoch *et al.*, 2012). Whereas the internal transcribed spacer (ITS) region has been suggested as the standard fungal barcode (Schoch *et al.*, 2012), this region is exceptionally variable for AMF and does not resolve closely related species (Stockinger *et al.*, 2010). Therefore, Stockinger *et al.* (2010) recommended a 1500 bp region, including a segment of the small subunit (SSU) rRNA gene, the entire ITS region, and a portion of the large subunit (LSU) rRNA gene for AMF DNA barcoding. However, 1500 bp is too long for NGS technologies. Therefore, the SSU region has been most commonly used for studying AMF communities with NGS methods (Öpik *et al.*, 2013). However, it is often difficult to compare results between studies, as there has been little consistency in the used target genes and primer sets. Moreover, each combination of target region and primer pair, with potentially different specificities and amplification kinetics (Kohout *et al.*, 2014), may bias the description of the fungal community sampled.

1.3 The benefits of the arbuscular mycorrhizal symbiosis for the host

The AMF association with the roots of the host will result in a range of benefits for the host. More specifically, the AMF will enhance P and N uptake, increase tolerance to abiotic stress, protect against biotic stress and improve soil structure and stability (Fig. 1.4).

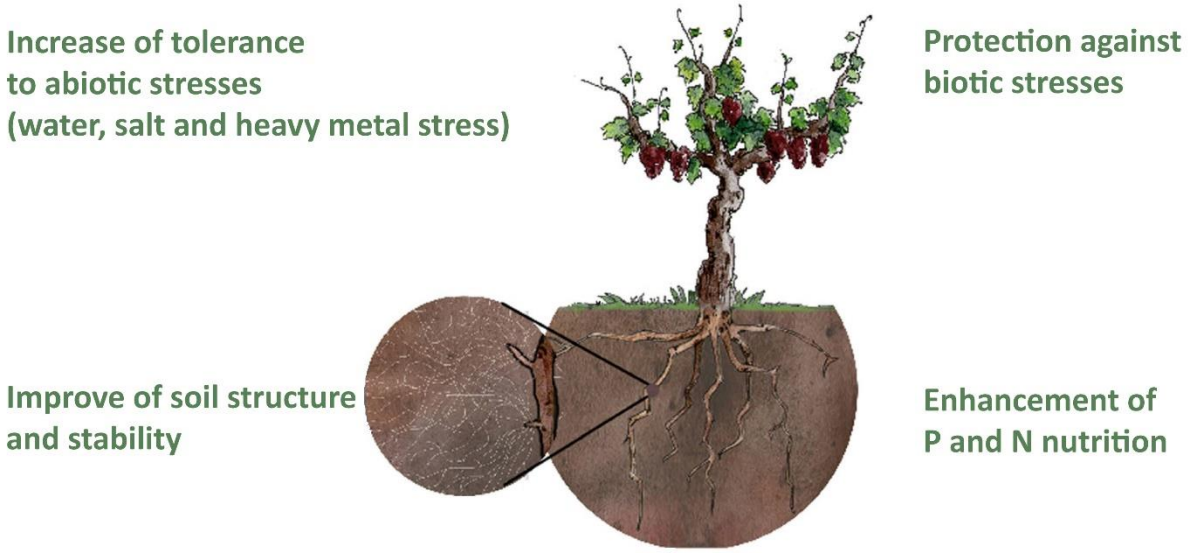


Figure 1.4 The association of AMF with the roots of the host will provide a range of benefits for the host. Adapted from Trouvelot *et al.* (2015).

1.3.1 Enhancement of nutrition

Phosphate

In the environment, phosphorus exist as inorganic orthophosphate, primarily bound with inert complexes with cations such as iron and aluminum phosphate, and as organic molecules, such as lecithin and phytate. Orthophosphate is the only form directly accessible to plants. Orthophosphate bound to cations or part of organic molecules is not directly available to the plant. As orthophosphate is negatively charged (PO_4^{-3}), it is easily sequestered by cations such as iron, aluminum and calcium (Vance *et al.*, 2003), making it highly immobile in the soil and leaving meager amounts of free orthophosphate in the soil solution. Consequently, the uptake of orthophosphate by the roots often generates a depletion zone surrounding the roots of the plant (Javot *et al.*, 2007).

Plants have a number of mechanisms to overcome low levels of orthophosphate. Next to increasing the root surface area with root hairs to maximize access to freely available orthophosphate and dissolving orthophosphate trapped in complexes with organic acids, plants can form symbiotic associations with AMF to benefit from their efficient phosphate acquisition capacity (Javot *et al.*, 2007). First, the extensive hyphal network can grow far beyond the reach of the root hairs and the phosphate depletion zone, providing an efficient nutrient-absorbing network (Jakobsen *et al.*, 1992). Second, the hyphal network can contribute to the release of phosphate from inorganic complexes with low solubility (Finlay, 2008). Third, the hyphae have an enhanced ability to mineralize organic phosphate, making also organic sources of phosphorus available to the host plant (Feng *et al.*, 2003).

Nitrogen

AMF can also play a major role in the uptake of nitrogen. Nitrogen is very mobile in the soil, especially under humid conditions, making it generally easier available to the plant. Under arid or very wet conditions when leaching can take place, the availability of nitrogen may be limited. AMF may help in the acquisition of nitrogen as the fungi are able to take up both nitrate and ammonium (Smith & Read, 2008). An ammonium transporter that might be involved in the nitrogen uptake by the extraradical hyphae has been characterized (Lopez-Pedrosa *et al.*, 2006). Besides inorganic nitrogen uptake, AMF can transfer substantial amounts of nitrogen to their host plant from organic material, which represents a large proportion of total soil nitrogen (Leigh *et al.*, 2009). It has been shown that AMF can accelerate the decomposition of organic material (Miransari, 2011) and even acquire recalcitrant and labile forms of organic nitrogen (Whiteside *et al.*, 2012).

Uptake of other nutrients

AMF can also help with the uptake of micronutrients. For example, Li *et al.* (1991) demonstrated hyphal uptake and translocation of copper to *Trifolium repens*, and that AMF contributed up to 62% of the total copper uptake. Additionally, in comparison to non-mycorrhizal grapevine, the uptake of calcium and potassium was increased in mycorrhizal grapevine (Nikolaou *et al.*, 2002).

1.3.2 Increase of tolerance to abiotic stresses

AMF have the potential to enhance the tolerance to abiotic stresses, such as drought, salinity or heavy metals stress. These abiotic stresses can cause extensive damage to agricultural production.

Water stress

The water relations of the host plant are modified by the AMF symbiosis. Plants colonized with AMF often have a higher resistance to drought stress. For example, is has been shown that inoculated tomato plants were more resistant against drought stress than non-inoculated plants (Dell'Amico *et al.*, 2002). Augé (2001) concluded that AMF effects on water relations included indirect effects that were related to changes in soil structure and plant nutrition, as well as direct effects. It is well known that AMF positively affect soil structure and aggregate stability (Rillig & Mummey, 2006), which can indirectly increase water availability to the plant. Also, limited nutrient availability under drought conditions can reduce root growth, restraining the accessibility of water. Therefore, enhancing the uptake of nutrients through the hyphae of AMF, which allows root growth, can indirectly contribute to water availability. For example, it has been shown that sorghum inoculated with AMF acquired more phosphorus from dry soil than non-inoculated plants (Neumann & George, 2004). More direct mechanisms that can enhance water uptake include the increase of root hydraulic conductivity (Koide, 1985) and extensive absorption of water by the extraradical hyphae (Faber *et al.*, 1991; Ruiz-Lozano & Azcon, 1995).

Salt stress

Salt stress occurs in soils with high soil salinity, which refers to a high amount of dissolved salts in the soil. It has become a major threat to plant growth and productivity, mainly in arid and semi-arid areas (Evelin *et al.*, 2009). High soil salinity can develop an osmotic stress that greatly impairs plant transpiration and photosynthesis, and induce changes in the mineral balance leading to metabolic dysfunctions and altered physiological mechanisms (Shannon & Grieve, 1999; Feng *et al.*, 2002). AMF naturally occur in saline environments (Garcia & Mendoza, 2007) and can alleviate salt stress in many host plants (Evelin *et al.*, 2009). The increase of tolerance to salt stress in plants colonized by AMF is probably due to the improvement of nutrient uptake (Giri *et al.*, 2003; Giri & Mukerji, 2004). In addition, AMF can improve water uptake (see above), diminishing the physiological drought caused by the salt stress (Ruiz-Lozano, 2003).

Tolerance to heavy metals

AMF have also been demonstrated to alleviate heavy metal stress (Hildebrandt *et al.*, 2007). For example, the mycelium network has a buffering effect on cadmium uptake, reducing the toxic effect of cadmium on plant growth (Rivera-Becerril *et al.*, 2002). It is thought that AMF immobilize heavy metals in their fungal biomass, protecting the roots of the host to heavy metal exposure (de Andrade *et al.*, 2008). Metals can adsorb to the hyphal walls of fungi, because chitin in the hyphal walls has a high metal-binding capacity, acting as barrier against metal transport (Christie *et al.*, 2004). Next to cadmium, also zinc and copper can accumulate in the cell wall of AMF (Gonzalez-Guerrero *et al.*, 2008). Also glomalin, a glycoprotein produced by AMF have metal-chelating properties, diminishing the metal availability for plants (Gonzalez-Chavez *et al.*, 2004).

1.3.3 Protection against biotic stresses

AMF can protect their host plant against fungal and nematode plant pathogens (Veresoglou & Rillig, 2012). AMF have not been shown to interact directly with pathogens through antagonism or mycoparasitism. However, Harrier and Watson (2004) proposed a number of indirect mechanisms through which AMF can reduce biotic stresses. First, AMF increases plant growth due to an improvement of the mineral nutrient status, which can decrease the susceptibility of the host to infection by pathogens (Bodker *et al.*, 1998). Second, AMF and fungal or nematode pathogens are directly competing for infection sites on the roots of the host. It has been shown that pathogens did not penetrate root cells already infected with AMF (Cordier *et al.*, 1996). Additionally, AMF and root pathogens may also directly compete for host-derived photosynthates (Azcon-Aguilar & Barea, 1996). Third, when AMF colonize the roots of the plant, an increase in cell wall lignification can occur, which can protect the roots from penetration by other pathogens (Schob 1999). Fourth, AMF colonization changes the deposition of root exudates, which can decrease the pH of the rhizosphere and negatively affect the migration of pathogens to the root system (Harrier & Watson, 2004). Fifth, AMF colonization alters the root respiration rate and the quality and quantity of the root exudates, which can change the microbial community composition of the rhizospere (Marschner *et al.*, 1997). The establishment of specific rhizosphere communities may have an important impact on the pathogenic organisms. Finally, after infection by AMF, plants can develop an enhanced defensive capacity. This mycorrhiza-induced resistance (MIR) shares characteristics with systemic acquired resistance (SAR) after pathogen infection and could provide protection against a wide range of biotrophic and necrotrophic pathogens, nematodes and herbivorous arthropods (Cameron *et al.*, 2013).

1.3.4 Improvement of soil structure and stability

AMF develop an extensive extraradical network into the surrounding soil, which can reach up to 30 meter of hyphae per gram of soil (Wilson *et al.*, 2009). Similarly to the action of roots, this hyphal network serves to entangle soil particles, organic materials and small aggregates, facilitating the formation of macroaggregates (Rillig & Mummey, 2006). Additionally, the soil protein glomalin produced by AMF also contributes to soil stability (Rillig *et al.*, 2002). It is thought to act as a sort of glue with hydrophobic properties. Therefore, a reduction in mycorrhizal biomass will result in a less soil stability, increasing the risk of soil erosion.

1.4 How arbuscular mycorrhizal diversity can contribute to ecosystem functioning

Biodiversity is the variety of life, including the variation of genes, species and functional traits. There is now unequivocal evidence that biodiversity increases ecosystem stability and contributes to ecosystem functions, such as capturing essential resources, producing biomass, decomposing and recycling biologically essential nutrients (Cardinale *et al.*, 2012). Since diversity indicators show rapid decline since 1970, and the rate of biodiversity loss does not appear to be slowing (Butchart *et al.*,

2010), the conservation of biodiversity receives attention from policy makers. The relation between biodiversity and ecosystem functioning provided additional arguments to preserve biodiversity.

Two ecological mechanisms explain the beneficial effect of biodiversity on ecosystem functioning and services. The first mechanism, known as the sampling effect, explains how increasing diversity also increases the chance of including key species with a high impact on ecosystem functioning or services (Tilman *et al.*, 1997; Turnbull *et al.*, 2013). The second mechanism, known as the complementarity effect, explains how a more diverse community can be expected to contain a higher functional diversity through complementarity, which leads to a more efficient exploitation of resources in the system (Loreau & Hector, 2001). Complementarity in plant communities, for example, arises between legumes, which have the ability to fix atmospheric nitrogen, and other plants, which have access only to soil nitrogen. Together, they will more efficiently exploit nitrogen, increasing the biomass production of the system.

Although the role of above-ground biodiversity in maintaining ecosystem functioning is relatively well studied (e.g. Balvanera *et al.*, 2006), below-ground diversity received substantially less attention. Yet, recent studies have demonstrated that important ecosystem processes are negatively affected by a loss in soil biodiversity (Wagg *et al.*, 2011). There is currently increasing evidence that AMF cover a broad range of functional types (Maherali & Klironomos, 2007), suggesting that they may strongly contribute to ecosystem functioning through the complementarity effect. First, AMF can differ in colonization strategies (Hart & Reader, 2005). For example, AMF from the Diversisporales start root colonization from spores, while AMF from the Glomerales can also start root colonization from hyphal fragments, making the latter more efficient colonizers (Hart & Reader, 2002). Second, the growth of the mycelium can differ considerably among species and also among isolates within a same species (Munkvold *et al.*, 2004). Third, AMF can also differ in their strategies to acquire phosphorus (Jansa *et al.*, 2005). For example, compared to colonization by a single AMF, colonization by two AMF with different spatial abilities to acquire soil phosphate induces a larger growth response in the host plant (Smith *et al.*, 2000). Therefore, high AMF diversity may result in a more efficient exploitation of soil resources and a loss in AMF diversity may negatively affect plant functionality and soil fertility (Jeffries *et al.*, 2003).

1.5 Arbuscular mycorrhizal fungi and conventional agriculture

The application of fertilizers, pesticides and irrigation in intensive agriculture has allowed to dramatically increase food production over the past 60 years. These agricultural practices, however, also cause severe environmental problems, including the degradation of water quality, soil eutrophication, loss of biodiversity and accumulation of pesticides (Tilman *et al.*, 2002). Conventional agriculture may also have detrimental effects on AMF.

1.5.1 Soil tillage

Soil tillage represent some form of intense soil disruption due to for example ploughing. As discussed above, AMF can develop an extensive hyphal network into the surrounding soil, which can reach up to tens of meters of hyphae per gram of soil (Wilson *et al.*, 2009). Repeated

destruction of this extensive hyphal network through soil tillage can radically alter the AMF communities in the soil. It has been shown that tillage decreases mycorrhiza diversity and can lead to dominance by taxa most tolerant to hyphal disruption (Jansa *et al.*, 2002). It has been hypothesized that intense tillage regimes favor AMF taxa that can colonize new plants through fragment hyphal networks and infect root-pieces (Hamel, 1996). For example, AMF from the Gigasporaceae family do not use hyphal fragments to colonize roots, and it has been reported that tillage decreases diversity in this family (Daniell *et al.*, 2001; Castillo *et al.*, 2006). In contrast, AMF in the Glomeraceae are able to randomly reconnect hyphae in close proximity after disruption. Therefore, they are able to recover rapidly after soil tillage and are present in most agricultural ecosystems (Rosendahl *et al.*, 2009).

1.5.2 Nutrient input

Nutrient exchange between the AMF and the host is the core of the symbiosis and reciprocal transfer of nutrients is an essential requirement for proper function. Therefore, it is not unexpected that the exchanged nutrients, such as phosphate, nitrogen and carbon, act as regulatory components of the symbiosis (Fitter, 2006; Javot *et al.*, 2007). Nutrient enrichment can improve the nutrient limitation of the host, making symbiotic partners costly or even parasitic (Johnson, 2010). High input of nutrients may severely decrease resource allocation to mycorrhizal roots, resulting in decreased AMF colonization (Mäder *et al.*, 2000). When the resource allocation to the roots is diminished, competition for limited carbon resources will increase. This may lead to shifts in the AMF community, favoring those AMF that are best at acquiring carbon sources from the host (Johnson, 2010).

1.5.3 Weeds

Because the AMF associated with different host plants can differ (Vandenkoornhuyse *et al.*, 2003), it is likely that there is a correlation between the diversity of the plant community and AMF community (Landis *et al.*, 2004). Therefore, weed control practices can have a large impact on the AMF community. Herbaceous weeds could promote different sets of dominant mycorrhizal fungi, potentially providing a wider spectrum of these fungi for colonizing the roots of the crop. Consequently, promoting plant diversity could also increase AMF diversity in agro-ecosystems. The AMF associated with weeds can also provide indirect benefits for the crop through for example improving soil structure and water availability (see above).

1.5.4 Pest management practices

In soil pest management, it is often difficult to reach an equilibrium between pest control and the protection of beneficial micro-organisms such as AMF. The fumigation of soils with nematocides or biocides may be necessary to alleviate disease problems caused by soil-born pests. However, when using these techniques, naturally occurring AMF in the soil may not be spared. For example, it has been shown that vines were weaker due to the decrease in AMF after fumigation with mythylbromide (Menge *et al.*, 1978). Additionally, Bharat (2011) showed that commonly used fungicides, such as carbendazim, mancozeb, copper sulphate and aureofungin, significantly

inhibited the AMF colonization in the roots of apple. They demonstrated that the application of mancozeb resulted in the highest reduction of AMF colonization, whereas carbendazim resulted in the lowest reduction. Finally, herbicides may have an indirect effect through elimination of weeds, which could otherwise act as AMF hosts (see 1.5.3).

1.6 Arbuscular mycorrhizal fungi and sustainable agriculture

Organic agriculture, proposed as a system that incorporates management procedures that coincide with natural processes and minimizes the human impact on the environment (European directive No. 2092/91), has been proposed as an alternative to conventional farming. Therefore, typical organic farming practices exclude the use chemical fertilizers, herbicides and pesticides and manage the soil trough addition of organic materials. Hence, organic farming is largely reliant on biological processes for the supply of nutrients and protection of crops. As a result, the soil microbial community is vital for the functioning of the agroecosystem and for the success of organic farming (Gosling *et al.*, 2006). Particularly AMF are important components of the soil microbial community in agricultural ecosystems and are considered as natural biofertilizers (see 1.3).

Phosphate is an essential mineral nutrient for plant growth and is one of the tree main mineral nutrients that is regularly applied in agriculture. However, phosphate sources are limited and it is estimated that most of the phosphate mines will be depleted in about 100 years (Cordell *et al.*, 2009; Gilbert, 2009). The excess application of phosphate fertilizers is also an important cause of water eutrophication. Therefore, an improvement of the phosphate uptake efficiency would greatly benefit sustainable agriculture. As discussed above (see 1.3.1), plants can form symbiotic associations with AMF to benefit from their efficient phosphate acquisition capacity. It has been estimated that inoculation with AMF could reduce phosphate input by 80% (Jakobsen, 1995).

AMF may also provide opportunities to increase the resilience of agro-ecosystems in a context of global change. The increasing occurrence of drought due to global warming is also a major problem for agriculture. The period from 1983 to 2012 was likely the warmest 30-year period of the last 1400 years in the Northern Hemisphere and it is very likely that heat waves will occur more often in large parts of Europe (IPCC, 2015). Also for Flanders, the probability and intensity of droughts is estimated to increase, while summer precipitation is expected to decline (VMM, 2009). This evolution towards drier and warmer summer will cause drought stress and yield losses due to water shortage. Therefore, AMF may become increasingly important as plants colonized with AMF have a higher resistance to drought stress (see 1.3.2).

To conclude, AMF may reconcile the need to produce more food in the next 50 year and the need to reduce the dependency of agriculture to agrochemicals. A systematic quantitative analysis of the response of crops to AMF inoculation, however, remains to be done. Additionally, little is known regarding the role of AMF specificity and the diversity of an AMF inoculum on crop growth. It is also important to acquire knowledge on the diversity and community composition of AMF in agricultural ecosystems, and on which environmental drivers mediate this diversity and community

composition. However, the studies researching this topic that used field scale investigations using trap cultures and microscopic analysis of spores, are limited in scale and likely do not provide full insight in true mycorrhizal diversity (Oehl *et al.*, 2004; Purin *et al.*, 2006). Others used real-time PCR or fingerprinting methods and thus likely lacked sufficient resolution to thoroughly characterize AMF diversity (e.g. Hazard *et al.*, 2013; Jansa *et al.*, 2014).

1.7 Aims and thesis outline

The general objective of this thesis is to gain a better understanding of how environmental factors, such as site geography, soil properties and management, influence the diversity and community composition of AMF in agricultural ecosystems (Fig. 1.5). We characterized AMF communities in apple orchards and vineyards, both important economical crops to address the following questions:

Which primer pairs, previously used in AMF studies, perform best for use in high-throughput sequencing-based AMF community analysis?
Which AMF occur in the roots of cultivated apple trees and grapevines?
How do AMF communities respond to environmental variation across apple orchards and vineyards?
Do organically managed orchards harbor more diverse AMF communities than conventionally managed orchards?
How do different amounts and forms of phosphorus fertilizer affect AMF communities in the roots of apple trees?
Are there differences in tolerance to phosphorus fertilization between AMF taxa?
What is the degree of AMF specificity to crop species?
What is the role of AMF diversity on crop growth?

In **chapter 2**, we evaluated six primer pairs targeting the nuclear rRNA operon for characterization of AMF communities using 454 pyrosequencing. Primer pairs were evaluated in terms of in silico coverage of Glomeromycota fungi, the number of high-quality sequences obtained, selectivity for AMF species, reproducibility and ability to accurately describe AMF communities.

In **chapter 3**, we studied the response of AMF in the roots of cultivated apple trees with increasing orchard fertilization. We characterized the diversity present in the apple roots and investigated which environmental factors drive AMF diversity and community composition.

In **chapter 4**, we experimentally tested whether different amounts and forms of phosphorus fertilizer affected AMF diversity and community composition associated with the roots of apple trees and identified differences in tolerance to phosphorus fertilization between AMF taxa.

In **chapter 5**, we focused on the AMF diversity present in vine roots and investigated which environmental factors, including the copper concentration in the soil, drive AMF diversity and community composition.

In **chapter 6**, we applied meta-analytical techniques on the literature reporting on the response of crop plants to AMF inoculations to assess the degree of arbuscular mycorrhizal specificity to crop species, and evaluate the role of AMF diversity on crop performance.

In **chapter 7**, we discuss the most important findings of this thesis. We conclude with some shortcomings and suggestions for future research regarding the diversity of AMF in agricultural ecosystems.

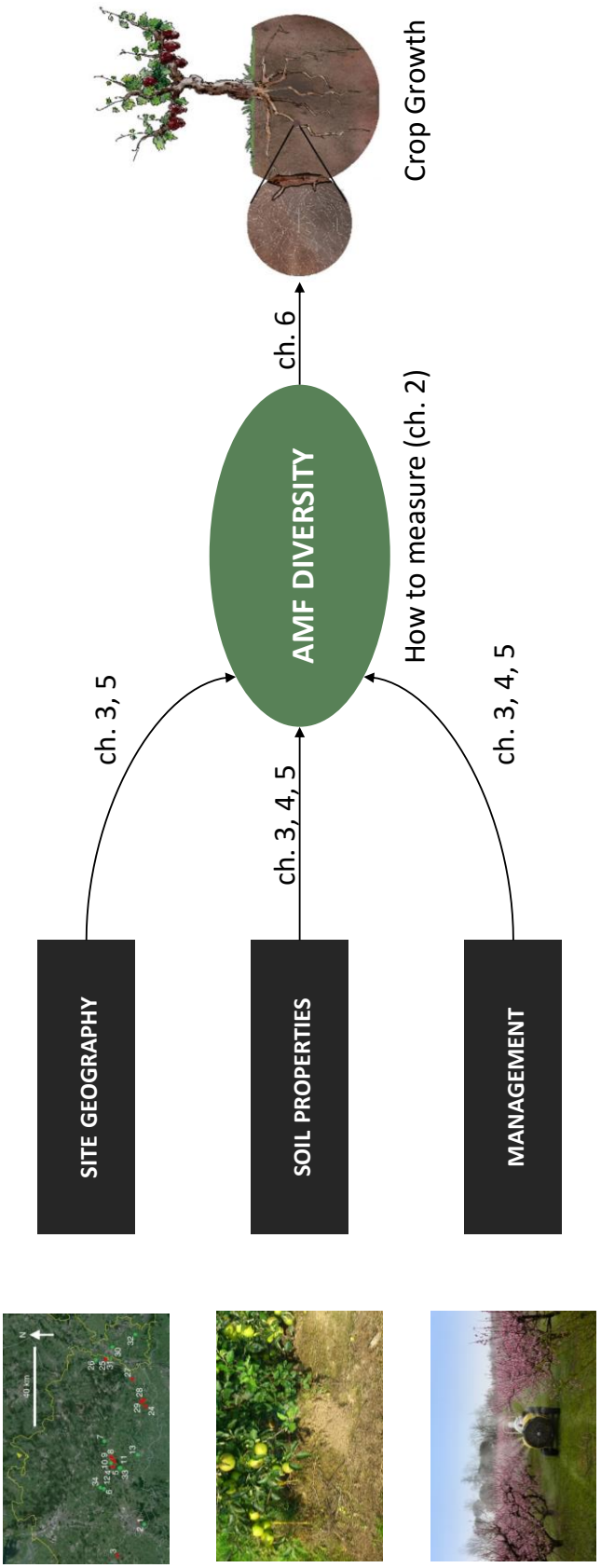


Figure 1.5 Schematic outline of the links studied across the different chapter of this thesis (ch.2-6).


4112728
WAUD
4180191
ML1-B
CCT ATC CCC TGT GT
Tm=69.3°C
13.0OD =
64752107
M.VAN GEEL
165429261
glo454-M1
5'- CCA TCT CAT
Tm=71.4°C
50.5OD =
64112732
M.WAUD
164180195
LSUmBr4-B
5'- CCT ATC CCC TG
Tm=69.4°C
12.3OD =

Chapter 2

Evaluation of six primer pairs targeting the nuclear rRNA operon for characterization of arbuscular mycorrhizal fungal communities using 454 pyrosequencing

Adapted from:

Van Geel Maarten, Busschaert Pieter, Honnay Olivier, Lievens Bart. 2014. Evaluation of six primer pairs targeting the nuclear rRNA operon for characterization of arbuscular mycorrhizal fungal (AMF) communities using 454 pyrosequencing. *Journal of Microbiological Methods* **106**: 93-100.

2.1 Summary

In the last few years, 454 pyrosequencing-based analysis of arbuscular mycorrhizal fungal (AMF; Glomeromycota) communities has tremendously increased our knowledge of the distribution and diversity of AMF. Nonetheless, comparing results between different studies is difficult, as different target genes (or regions thereof) and primer combinations, with potentially dissimilar specificities and efficacies, are being utilized. In this study we evaluated six primer pairs that have previously been used in AMF studies (NS31-AM1, AMV4.5NF-AMDGR, AML1-AML2, NS31-AML2, FLR3-LSUmBr and Glo454-NDL22) for their use in 454 pyrosequencing based on both an *in silico* approach and 454 pyrosequencing of AMF communities from apple tree roots. Primers were evaluated in terms of (i) *in silico* coverage of Glomeromycota fungi, (ii) the number of high-quality sequences obtained, (iii) selectivity for AMF species, (iv) reproducibility and (v) ability to accurately describe AMF communities. We show that primer pairs AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 outperformed the other tested primer pairs in terms of number of Glomeromycota reads (AMF specificity and coverage). Additionally, these primer pairs were found to have no or only few mismatches to AMF sequences and were able to consistently describe AMF communities from apple roots. However, whereas most high-quality AMF sequences were obtained for AMV4.5NF-AMDGR, our results also suggest that this primer pair favored amplification of Glomeraceae sequences at the expense of Ambisporaceae, Claroideoglomeraceae and Paraglomeraceae sequences. Furthermore, we demonstrate the complementary specificity of AMV4.5NF-AMDGR with AML1-AML2, and of AMV4.5NF-AMDGR with NS31-AML2, making these primer combinations highly suitable for tandem use in covering the diversity of AMF communities.

2.2 Introduction

Arbuscular mycorrhizal fungi (AMF) form a root symbiosis with approximately 80% of the terrestrial plant species and improve nutrient and water uptake as well as pathogen resistance in their hosts in exchange for plant assimilated carbon (Smith & Read, 2008). It is therefore increasingly acknowledged that AMF play a key role in ecosystem functioning, and therefore, quantifying and understanding their distribution and diversity is of major importance (Rillig & Mummey, 2006; van der Heijden *et al.*, 2008). All AMF belong to the phylum Glomeromycota (Schüssler *et al.*, 2001), which is divided in four orders (Diversisporales, Glomerales, Archaeosporales and Paraglomerales) and ten families. Most AMF species belong to the families of Diversisporaceae, Acaulosporaceae, Gigasporaceae, Claroideoglomeraceae and Archaeosporaceae (Redecker et al., 2013).

Molecular methods have become the standard for studying AMF communities (Gorzelak *et al.*, 2012). Especially high throughput sequencing technologies such as 454 amplicon pyrosequencing (Margulies *et al.*, 2005), enabling highly efficient characterization of microbial communities by sequencing medium-sized (200-600bp) amplicons are currently often used (e.g. Öpik et al., 2009, 2013; Lekberg *et al.*, 2012; De Beenhouwer *et al.*, 2014). For molecular identification of AMF, the nuclear ribosomal RNA (rRNA) operon is commonly used, mainly due to its high resolving power and alignment capability across broad taxonomic groups (Stockinger *et al.*, 2010; Schoch *et al.*, 2012). Whereas the internal transcribed spacer (ITS) region has been suggested as the standard fungal barcode (Schoch *et al.*, 2012), this region is exceptionally variable for AMF and does not resolve closely related species (Stockinger *et al.*, 2010). Therefore, Stockinger *et al.* (2010) recommended a 1500 bp region, including a segment of the small subunit (SSU) rRNA gene, the entire ITS region, and a portion of the large subunit (LSU) rRNA gene for AMF DNA barcoding. However, so far the SSU region has been most commonly used for studying AMF communities (Öpik *et al.*, 2013).

Different primer pairs have been used to amplify different parts of the SSU region to investigate AMF communities. Öpik *et al.* (2009) were the first studying AMF communities using 454 pyrosequencing and used the NS31-AM1 primer pair, one of the first primer pairs designed for the detection and identification of AMF (Simon *et al.*, 1992; Helgason *et al.*, 1998). Whereas this primer pair has been frequently used in AMF studies, it was also shown to pick up non-AMF species and to not detect all species from the basal AMF families Ambisporaceae, Archaeosporaceae and Paraglomeraceae (Daniell *et al.*, 2001). Lumini *et al.* (2010) used AMV4.5NF and AMDGR (Sato *et al.*, 2005) for 454 pyrosequencing-based AMF investigation, and showed that this primer pair resulted in a higher proportion of AMF sequences and detection of a broader spectrum of Glomeromycota when compared to NS31-AM1. In order to overcome the disadvantages of the NS31-AM1 primer pair, Lee *et al.* (2008) designed primer pair AML1-AML2 showing a better *in silico* specificity and coverage across the Glomeromycota. For 454 pyrosequencing-based AMF studies, the reverse primer AML2 has been combined with NS31 (Davison et al., 2012).

In addition to the SSU region, segments of the LSU region have also been used for studying AMF communities using pyrosequencing. More specifically, Stockinger *et al.* (2010) recommended the LSU-D2 region, marked by the primers FLR3 and LSUmBr. Lekberg *et al.* (2012) exploited this region to study shifts in AMF communities using a novel forward primer, Glo454, in combination with the reverse primer NDL22 designed by van Tuinen *et al.* (1998).

AMF community analysis using next-generation sequencing has tremendously increased our knowledge of the diversity and the spatial and temporal variation of AMF communities. However, comparing results between studies is often difficult, as there has been little consistency in target genes and primer sets utilized. Moreover, each combination of target region and primer pair, with potentially different specificities and amplification kinetics (Kohout *et al.*, 2014), may bias the description of the fungal community sampled. Therefore, the objective of this study was to critically evaluate the performance of six different AMF primer pairs, previously used in AMF studies, for use in high-throughput sequencing-based AMF community analysis. Different regions of the rRNA gene were evaluated *in silico* with respect to their effectiveness to resolve AMF species; the *in silico* coverage of Glomeromycota was assessed; and the primer pairs were compared by evaluating their ability to characterize AMF communities in field samples using 454 pyrosequencing. Primers were evaluated with respect to (i) the number of high-quality sequences obtained, (ii) selectivity for AMF species, (iii) reproducibility and (iv) ability to accurately describe AMF communities. In the framework of an ongoing study on the distribution of AMF across apple orchards of different management types, we focused on the AMF communities of cultivated and wild apple trees in the central and eastern part of Belgium. Previous work has shown that cultivated apple trees harbor a high diversity of AMF species and genera (Purin *et al.*, 2006).

2.3 Materials and Methods

2.3.1 Primer selection

Six SSU- or LSU-targeting primer pairs, commonly used in AMF studies, were selected for this study (Table 2.1; Fig. 2.1). Primer combinations included four primer pairs previously used in 454 pyrosequencing-based AMF community analyses: NS31-AM1, AMV4.5NF-AMDGR, NS31-AML2 and Glo454-NDL22. Additionally, we included FLR3-LSUmBr and AML1-AML2 in our comparison.

Table 2.1 Primer pairs used in this study.

	Primer pair[a]	Sequence (5′ → 3′)	Fragment size	Target region[b]	Reference
1.	NS31 (F) AM1 (R)	TTGGAGGGCAAGTCTGGTGCC GTTTCCCGTAAGGCGCCGAA	550 bp	SSU	Simon (1992) Helgason (1998)
2.	AMV4.5NF (F) AMDGR (R)	AAGCTCGTAGTTGAATTTCG CCCAACTATCCCTATTAATCAT	300 bp	SSU	Sato *et al.* (2005) Sato *et al.* (2005)
3.	AML1 (F) AML2 (R)	ATCAACTTTCGATGGTAGGATAGA GAACCCAAACACTTTGGTTTCC	800 bp	SSU	Lee *et al.* (2008) Lee *et al.* (2008)
4.	NS31 (F) AML2 (R)	TTGGAGGGCAAGTCTGGTGCC GAACCCAAACACTTTGGTTTCC	550 bp	SSU	Simon (1992) Lee *et al.* (2008)
5.	FLR3 (F) LSUmBr1 (R) LSUmBr2 (R) LSUmBr3 (R) LSUmBr4 (R) LSUmBr5 (R)	TTGAAAGGGAAACGATTGAAGT DAACACTCGCATATATGTTAGA AACACTCGCACACATGTTAGA AACACTCGCATACATGTTAGA AAACACTCGCACATATGTTAGA AACACTCGCATATATGCTAGA	370-436 bp	LSU	Kruger *et al.* (2009) Kruger *et al.* (2009) Kruger *et al.* (2009) Kruger *et al.* (2009) Kruger *et al.* (2009) Kruger *et al.* (2009)
6.	Glo454 (F) NDL22 (R)	TGAAAGGGAAACGATTGAAGT TGGTCCGTGTTTCAAGACG	350 bp	LSU	Lekberg *et al.* (2012) van Tuinen *et al.* (1998)

[a] F, Forward primer; R, Reverse primer.
[b] SSU, small subunit rRNA gene; LSU, large subunit rRNA gene.

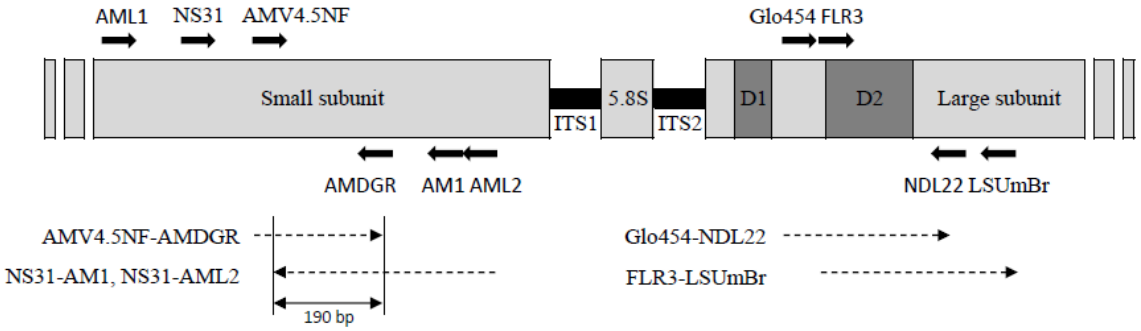


Figure 2.1 Schematic map indicating the target regions of the rRNA gene and primer locations. Dashed lines represent the sequenced regions and arrows indicate the direction of sequencing.

2.3.2 In silico target region evaluation

In order to assess the capacity of the different rRNA gene regions targeted by the selected primers to distinguish different AMF species, the nucleotide diversity (Pi) was calculated using 458 (SSU) and 614 (LSU) aligned AMF reference sequences belonging to almost 100 AMF species (retrieved from Krüger *et al.*, 2012). Pi is the average number of nucleotide differences in a 10 base window between two sequences (Nei, 1987).

2.3.3 In silico primer evaluation

In order to assess the ability of the selected primers to efficiently and specifically amplify AMF sequences, all primer sequences were individually subjected to an *in silico* analysis using the PrimerProspector software (Walters *et al.*, 2011). Each primer sequence was screened against all sequences from the Krüger *et al.* (2012) database, representing the ten major families in the Glomeromycota, including the Acaulosporaceae, Ambisporaceae, Archaeosporaceae, Claroideoglomeraceae, Diversisporaceae, Glomeraceae, Geosiphonoceae, Gigasporaceae, Pacisporaceae and Paraglomeraceae. PrimerProspector scores were calculated as [non-3' mismatches * 0.40] + [3' mismatches * 1.00] + [3' terminus mismatch = True, + 3.00] + [non 3' gaps * 1.00] + [3' gaps * 3.00], giving larger penalties to gaps and mismatches in the 3' end (last 5 bp) of the primer, which represents the most crucial region for primer extension (Lefever *et al.*, 2013). For each primer, the proportion of sequences having a good (<1), mediocre (≥1 and <2) and poor (≥2) score was calculated. In this regard, sequences representing the different AMF families were equally weighted to evaluate the primers at the family level rather than at the level of individual isolates.

2.3.4 Primer pair evaluation by assessing field samples using 454 pyrosequencing

Five commercial apple (*Malus domestica*; cv. 'Jonagold') orchards from the central and eastern part of Flanders, Belgium, were selected for this study. Additionally, wild apple trees (*Malus sylvestris*) from Meerdaalwoud, a forest ten kilometers south of Leuven (Belgium) were included in the study. All trees in the commercial orchards were grafted on M9 rootstocks. In August 2012, roots from three randomly chosen apple trees per sampling site were excavated, and the fine roots were collected, as these are especially known to contain AMF (Smith & Read, 2008) (Table 2.2).

Table 2.2 Orchards sampled in this study.

Orchard ID	Location	Latitude, Longitude	Sample ID
Bi	Oetingen	50°46'25"N, 4°4'34"E	Bi1, Bi2, Bi3
Ca	Nieuwerkerken	50°52'18"N, 5°11'33"E	Ca1, Ca2, Ca3
Ha	Herk-de-Stad	50°56'19"N, 5°10'6"E	Ha1, Ha2,Ha3
Hu	Ranst	51°10'13"N, 4°36'1.23"E	Hu1, Hu2, Hu3
Lo	Heers	50°45'13"N, 5°18'07"E	Lo1, Lo2, Lo3
Wi[a]	Meerdaalwoud	50°48'19"N, 4°41'17"E	Wi1, Wi2, Wi3

[a]Representing wild apple trees in a forest.

The roots were cut in 1-2 cm pieces and rinsed twice with sterile distilled water. Subsequently, DNA was extracted from *c.* 0.25 g root material using the UltraClean Plant DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer's instructions, and 10 times diluted prior to PCR amplification. 'Fusion' primers, required for the 454 process, were designed according to the guidelines for 454 GS-FLX Titanium Lib-L sequencing containing the Roche 454 pyrosequencing adapters and a sample-specific MID barcode in between each adapter and primer sequence (Appendix Table A1). In order to obtain sequences that could be compared between different primer sets, fusion primers were constructed in such a way that amplicons generated with NS31-AM1, AML1-AML2 and NS31-AML2 were sequenced from the reverse primer,

whereas the other amplicons were sequenced in the forward direction (Fig. 2.1; Appendix Table A1). PCR reactions were performed on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) in a reaction volume of 20 µl, containing 0.15 mM of each dNTP, 0.5 µM of each primer, 1x Titanium *Taq* PCR buffer, 1U Titanium *Taq* DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), and 1 µl genomic DNA. Before amplification, DNA samples were denatured at 94°C for 2 min. Next, 35 cycles were run, consisting of 45 s at 94°C, 45 s at 65°C and 45 s at 72°C, followed by a final elongation of 10 min at 72°C. For NS31-AM1, the conditions as described in Öpik *et al.* (2009) were used. After resolving the amplicons by agarose gel electrophoresis, amplicons within the appropriate size range were cut from the gel and purified using the Qiaquick gel extraction kit (Qiagen, Hamburg, Germany). Purified dsDNA amplicons were quantified using the Quant-iT PicoGreen® dsDNA Assay Kit and the Qubit fluorometer (both from Invitrogen, Gent, Belgium). PCR amplifications were performed twice for each primer pair to verify reproducibility. Subsequently, for each primer pair, amplicons were pooled in equimolar quantities, resulting in six amplicon libraries (one for each primer pair). The quality of the amplicon libraries was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). Each amplicon library was loaded onto a separate $1/8^{th}$ of a 454 Pico Titer Plate (PTP) and pyrosequencing was performed using the Roche GS-FLX instrument and Titanium chemistry according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany)

Irrespective of the target gene and targeted region within the gene, sequences obtained from the 454 pyrosequencing run were clustered into operational taxonomic units (OTUs) defined at 97% sequence similarity. This was particularly done to facilitate the downstream processing of all data obtained. Additionally, in previous studies the same sequence identity level was considered a reliable threshold to discriminate possible AMF species based on SSU or LSU sequences (Lumini *et al.*, 2010). However, it should be noted that species-level OTU cut-offs applied to different regions are probably better inferred from the analysis of sequence variability within the targeted region (see also further; Fig. 2.2). Clustering was performed with the Uparse algorithm, following the recommended pipeline (Edgar, 2013). Quality filtering of the reads was done with the 'fastq-filter' command allowing a maximum expected error of 0.5 for individual sequences. In order to maximize the number and length of retained sequences from the shorter amplicons, truncation length was set to 220, 250 and 210 bp for AMV4.5NF-AMDGR, FLR3-LSUmBr and Glo454-NDL22, respectively. For NS31-AM1, AML1-AML2 and NS31-AML2 obtained reads were truncated at 200 bp to be able to compare them to the shorter amplicons and at 400 bp for the analysis of the overlapping fragment (see later). Global singletons, i.e. OTUs represented by only a single sequence in the entire dataset, were removed prior to clustering as this improves the accuracy of diversity estimates (Waud *et al.*, 2014), as were chimeric sequences. OTUs were identified by querying the representative sequences (selected by Uparse) against GenBank using the BLAST algorithm (Altschul *et al.*, 1990). Taxonomic assignments were considered reliable when a ≥200 BLAST score value was found (Lumini *et al.*, 2010). Sequences with BLAST scores lower than 200 were classified as 'unknown'. Obtained identities were used to determine the distribution of the obtained sequences over the major five

fungal phyla (Glomeromycota, Ascomycota, Basidiomycota, Zygomycota and Chytridiomycota) allowing to assess the primer pair's selectivity towards Glomeromycota.

As the primer pairs NS31-AM1, FLR3-LSUmBr and Glo454-NDL22 yielded a low number of AMF sequences, these primer pairs were discarded from further analysis. In order to make a fair 'like for like' comparison of the remaining three primer pairs further evaluation was based on (i) the same samples, (ii) the same target region and (iii) the same amount of AMF sequences per sample (>350 sequences per sample). Hence, the analysis was restricted to 8 out of the 16 samples studied (Hu1, Bi1, Ca2, Ca3, Lo1, Lo2, Lo3, Wi1), as these were the only samples resulting in sufficient AMF sequences for all three primer pairs. Sequences obtained with the AMV4.5NF-AMDGR primer pair were trimmed to 220 bp and sequences originating from the AML1-AML2 and NS31-AML2 primer pairs were trimmed to 400 bp, resulting in an overlapping fragment of *c.* 190 bp between the reads obtained from the different primer pairs (Fig. 2.1). The resulting overlapping sequences were clustered in OTUs defined at 97% sequence similarity as described above. Representative sequences from each OTU were deposited in Genbank under the accession numbers KF776958-KF776996. Finally, samples were rarified to 350 AMF sequences per sample to perform further statistical analyses, resulting in a total of 5600 AMF sequences for each primer pair (8 samples; 2 replicates; 350 AMF sequences per sample). First, in order to assess primer pair reproducibility, pairwise Bray-Curtis, Jaccard en Sorensen dissimilarity indices were calculated in R (Vegan package, Oksanen *et al.*, 2013) for the two technical replicates and compared using an Analysis of Variance (ANOVA) model. Next, in order to compare the AMF communities obtained with these three primer pairs, non-metrical multidimensional scaling (NMDS) using Bray-Curtis distances was performed in R (Vegan package, Oksanen *et al.*, 2013). Additionally, the number of shared OTUs between the different primer pairs was calculated in Mothur (Schloss *et al.*, 2009) using the 'Venn' command. Further, OTU richness (i.e. the number of OTUs) and evenness (calculated in EstimateS, Colwell, 2006) per sample was compared for the different primer pairs using linear mixed models. 'Sample' was included as a random factor to account for between-sample variation. To accurately identify the obtained AMF OTUs, the representative sequence for each OTU was queried against the Krüger *et al.* (2012) database using the BLAST algorithm. This curated database was used as public databases such as Genbank may contain sequences of incorrectly identified AMF isolates (Schüssler *et al.*, 2003; Bidartondo, 2008). In contrast, the Krüger *et al.* (2012) database contains high-quality sequences, obtained from well characterized isolates according to the taxonomy of Schüssler and Walker (2010). To assess bias towards certain AMF families, the relative abundance of each Glomeromycota family per sample was compared between the different primer pairs using linear mixed models. Again, 'Sample' was included as a random factor to account for between-sample variation. All statistical analyses were performed in SPSS 20.0 (SPSS Inc., Chicago, IL).

2.4 Results

2.4.1 In silico target region evaluation

Four SSU regions with a high nucleotide diversity were found, the most variable of which is flanked by the primer pair AMV4.5NF-AMDGR (Fig. 2.2a). Although amplicons generated by AML1-AML2, NS31-AML2 and NS31-AM1 contain a portion of this variable region, they also include a less variable downstream segment. Additionally, AML1-AML2 amplifies another relatively variable region at the 5' end of the amplicon. Regarding the LSU region, both selected primer pair (FLR3-LSUmBr and Glo454-NDL22) amplify a region with a consistent level of high nucleotide diversity (Fig. 2. 2b).

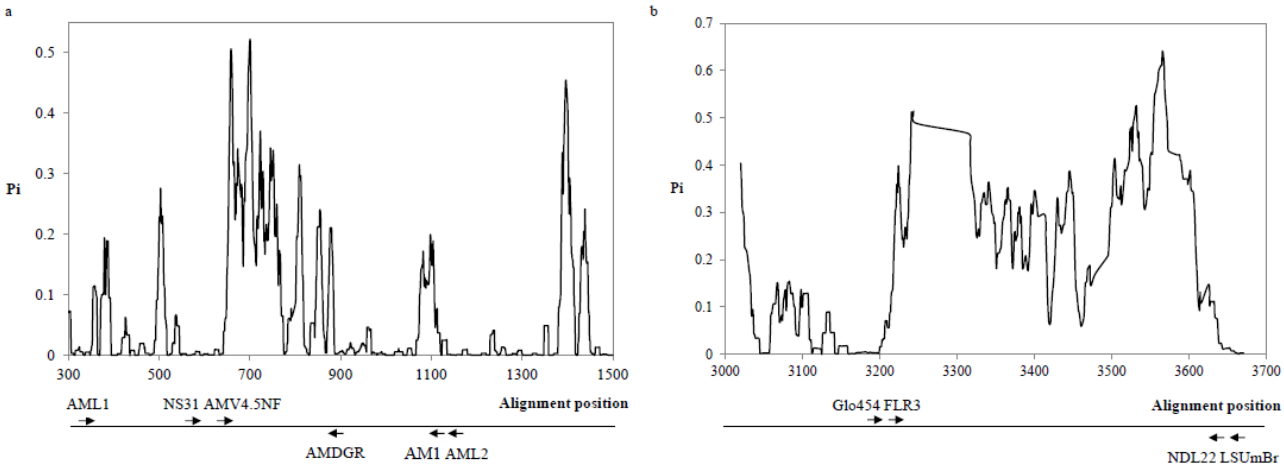


Figure 2.2 Nucleotide diversity (Pi) in the small subunit (SSU) (a) and large subunit (LSU) (b) ribosomal RNA gene of AMF fungi. Analysis of the SSU and LSU locus is based on 458 and 614 aligned sequences, respectively, from the Krüger et al. (2012) database, representing sequences from all Glomeromycota families. Pi is defined as the average number of differences in a 10 base window between sequences compared pairwise. The position of the primers used in this study is indicated relative to the alignment.

2.4.2 In silico primer evaluation

In order to assess the ability of the primers to detect as many AMF species as possible, all selected primers were screened *in silico* against a large set of Glomeromycota sequences (Fig. 2.3; Appendix Table A2). The primers obtaining the best (i.e., lowest) overall PrimerProspector scores, weighted at 100 sequences per family, were NS31 and NDL22, both developed to detect a wide range of fungi, and AMDGR and AML2, both particularly designed for AMF specificity (Fig. 2.3). These primers showed perfect matches to almost all Glomeromycota sequences tested. Generally, good overall scores (<1) were obtained for the other primer pairs, with the majority of primer sequences obtaining a score lower than 1 (Fig. 2.3). For FLR3, AM1 and AMV4.5NF a score <1 was obtained for approximately 90%, 80% and 70% of all family-weighted sequences tested, respectively. For the rest of the sequences a score between 1 and 2 was obtained (Fig. 2.3), mainly caused by the occurrence of mismatches inside and/or outside the 5 bp 3' end region of the primer, but not at the critical 3' terminal base (Appendix Table A2). For example, this was the case for FLR3 and many sequences belonging to the families Acaulosporaceae and Glomeraceae. For AM1, the mediocre score was mainly due to sequences from the families Claroideoglomeraceae and Gigasporaceae. The

AMV4.5NF primer showed, in addition to mismatches with sequences from the families Claroideoglomeraceae and Gigasporaceae in the 3' primer end, mismatches in the 3' primer region of sequences from the Glomeraceae, Pacisporaceae and Diversisporaceae (Appendix Table A2). For AML1 and Glo454, approximately 10% and 25% of all family-weighted sequences tested showed a poor PrimerProspector score (≥2), respectively, especially due to mismatches in the 3' terminus of the primer (Fig. 2.3). For the AML1 primer, this poor score was mainly due to the three Archaeosporaceae sequences tested, having a mismatch at the final 3' end of the primer, accounting for the 10% of family-weighted sequences having a score of 2 or more. For Glo454, mismatches in the 3' terminus could be found within a substantial portion of sequences belonging to diverse families, including Ambisporaceae, Claroideoglomeraceae, Diversisporaceae, Geosiphonoceae and Glomeraceae. Relatively poor PrimerProspector scores were obtained for LSUmBr1-5 primers sequences when screened individually (Fig. 2.3). However, these primers showed additive complementarity when reviewed in combination (as they are applied in practice), demonstrating greater potential for the detection of multiple AMF families (Appendix Table A2).

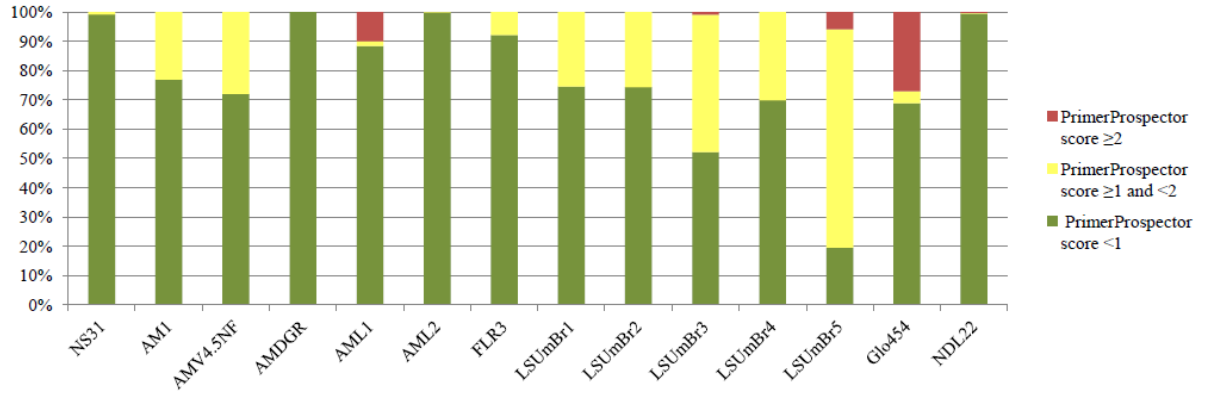


Figure 2.3 Results of PrimerProspector analysis of each primer sequence against sequences from the Krüger et al. (2012) database, giving larger penalties to mismatches in the 3' end of the primer. The proportion of sequences having a good (<1), mediocre (≥1 and <2) and poor (≥2) PrimerProspector score are presented. Proportions were equally weighted for every Glomeromycota family taken into account (refer to Appendix Table A2 for data on individual fungal families).

2.4.3 Number of sequences obtained using 454 pyrosequencing and selectivity towards Glomeromycota

In general, amplicons were obtained for each root sample (two replicates) tested for each primer pair. However, no PCR products were obtained for samples Ha1, Ha2 and Hu3 using primer pairs AML1-AML2 and NS31-AML2, even after several attempts (Appendix Table A3). The total number of sequences obtained per primer pair (amplicon library) varied between 139 190 for NS31-AM1 and 173 086 for NS31-AML2, with an overall average of 154 665 sequences per primer pair (Table 2.3). Between 36.1% and 92.3% of the total number of sequences passed quality control when sequences were truncated to a length of 200-250 bp. Notably, while the highest number of sequences was obtained for NS31-AML2, the proportion of retained sequences was relatively low (36.1%).

Table 2.3 Number of obtained sequences before and after sequence truncation.

Primer pair	Total No. sequences	Truncation length (bp)[a]	No. high-quality sequences
AMV4.5NF-AMDGR	156857	220	96179 (61.3%)
FLR3-LSUmBr	142734	250	131722 (92.3%)
Glo454-NDL22	158003	210	96999 (61.4%)
NS31-AM1	139190	200	63637 (45.7%)
		400	5276 (3.8%)
AML1-AML2	158122	200	73369 (46.4%)
		400	8720 (5.5%)
NS31-AML2	173086	200	62427 (36.1%)
		400	14831 (8.6%)

[a]For Glo454-NDL22, AMV4.5NF-AMDGR and FLR3-LSUmBr obtained reads were trimmed to 210, 220 and 250 bp, respectively, to maximize the number and length of retained high-quality sequences. For NS31-AM1, AML1-AML2 and NS31-AML2 obtained reads were truncated at 200 bp to be able to compare them to the shorter amplicons and at 400 bp for the analysis of the overlapping fragment.

The selectivity of the primer pairs towards Glomeromycota was assessed by examining the distribution of all retained high-quality sequences over the five major fungal phyla (Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota) (Table 2.4). All sequences obtained with AML1-AML2 represented members of the Glomeromycota, suggesting an extremely high AMF specificity for this primer pair. For AMV4.5NF-AMDGR and NS31-AML2, about three fourth of the sequences corresponded to Glomeromycota. Other sequences obtained with AMV4.5NF-AMDGR were mostly associated with Basidiomycota (11.1%) and Chytridiomycota (4.3%). Apart from Glomeromycota sequences, NS31-AML2 generated a vast amount of sequences associated with other eukaryotes, mainly nematodes (Table 2.4). In contrast, only few sequences were generated belonging to the Glomeromycota with the primer pairs NS31-AM1 (less than 1%), FLR3-LSUmBr (3.4%) and Glo454-NDL22 (0.3%). The majority of sequences obtained with NS31-AM1 belonged to the Ascomycota, while the majority of sequences generated with the primer pairs FLR3-LSUmBr and Glo454-NDL22 belonged to the Basidiomycota (86.3% and 92.0%, respectively).

Table 2.4 Distribution of retained high-quality sequences over the five major fungal phyla, as obtained with the six different primer pairs evaluated in this study.

	NS31-AM1 (200 bp)		NS31-AM1 (400 bp)		AMV4.5NF-AMDGR (220 bp)		AML1-AML2 (200 bp)		AML1-AML2 (400 bp)		NS31-AML2 (200 bp)		NS31-AML2 (400 bp)		FLR3-LSUmBr (250 bp)		Glo454-NDL22 (210 bp)	
Phylum	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Glomeromycota	602	0.95	29	0.55	69663	72.43	73369	100.00	8720	100.00	47992	76.88	12462	84.03	4423	3.36	324	0.33
Ascomycota	45220	71.06	2951	55.93	55	0.06	0	0.00	0	0.00	67	0.11	0	0.00	2580	1.96	2306	2.38
Basidiomycota	28	0.04	0	0.00	10712	11.14	0	0.00	0	0.00	0	0.00	0	0.00	113662	86.29	89197	91.96
Zygomycota	14	0.02	0	0.00	442	0.46	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Chytridiomycota	14	0.02	0	0.00	4176	4.34	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Other Eukaryotes	3150	4.95	308	5.84	1329	1.38	0	0.00	0	0.00	9466	15.16	1795	12.10	0	0.00	0	0.00
Unknown	14609	22.96	1988	37.68	9802	10.19	0	0.00	0	0.00	4902	7.85	574	3.87	11057	8.39	5172	5.33
Total	63637		5276		96179		73369		8720		62427		14831		131722		96999	

In total, AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 yielded 69 663, 73 369 and 47 992 high-quality Glomeromycota sequences of 200-220 bp, respectively (Table 2.4). On average 1990, 2717 and 1600 sequences per sample (replicates separated) were obtained with these primer pairs, respectively (Appendix Table A4). No significant differences in the number of sequences obtained per sample was observed between the primer pairs (F=1.05, P=0.35). When considering reads of 400 bp, only a fraction of the sequences was retained in the analysis (Table 2.3). However, little or no effect was seen on the distribution across taxonomic groups (Table 2.4), justifying performance of AMF diversity, community composition and reproducibility analyses using these reads.

2.4.4 AMF community composition, reproducibility and diversity

Further evaluation of AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2, focusing on differential AMF retrieval and primer pair reproducibility, was performed on an overlapping SSU fragment of 190 bp, using eight samples (two replicates), and a fixed number of 350 sequences per sample. No significant differences were observed between the composition of the AMF communities obtained with the different primer pairs (P=0.92; Appendix Fig. A2). On average, Bray-Curtis, Jaccard and Sorensen dissimilarity indices between the different replicates of 0.053, 0.299 and 0.184 (AMV4.5NF-AMDGR), 0.056, 0.310 and 0.200 (AML1-AML2), and 0.072, 0.350 and 0.238 (NS31-AML2) were found, respectively. No significant differences in the dissimilarity indices between both replicates were observed between the primer pairs (Bray-Curtis F=0.77, P=0.48; Jaccard F=0.13, P=0.88; Sorensen F=0.26, P=0.76), suggesting that all three primer pairs produce reproducible AMF fingerprints.

In total, 33, 26 and 28 AMF OTUs were obtained with the AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 primer pairs, respectively (Appendix Table A5). Rarefaction curves, assessing the OTU richness per primer pair, were generally tending towards saturation (Appendix Fig. A1), suggesting that our sequencing depth was sufficient to accurately detect the majority of AMF. Twenty OTUs were shared between the different primer pairs, while 25 OTUs were shared between AMV4.5NF-AMDGR and NS31-AML2, 21 between AMV4.5NF-AMDGR and AML1-AML2 and 23 between AML1-AML2 and NS31-AML2 (Fig. 2.4). Out of the 33 OTUs obtained with AMV4.5NF-AMDGR, 7 OTUs (5 Glomeraceae and 2 Claroideoglomeraceae OTUs) were unique to this primer pair, while 2 (1 Glomeraceae and 1 Diversisporaceae OTU) out of 26 OTUs were unique to the AML1-AML2 primer pair (Fig. 2.4). The obtained number of OTUs was not significantly different between the three primer pairs (F=0.26, P=0.73) (Fig. 2.5). However, when results from different primer pairs (also rarified to 350 AMF sequences per sample) were combined, the combination of AMV4.5NF-AMDGR and AML1-AML2 and the combination of AMV4.5NF-AMDGR and NS31-AML2 did result in a higher number of OTUs per sample, whereas the combination of AML1-AML2 and NS31-AML2 did not (Fig. 2.5). The combination of all three primer pairs, did not result in significantly more OTUs than observed for AMV4.5NF-AMDGR + AML1-AML2 and AMV4.5NF-AMDGR + NS31-AML2 (Fig. 2.5). The evenness of the AMF communities obtained with AMV4.5NF-AMDGR was found to be significantly lower than the evenness of the other (combinations of) primer pairs (F=5.36, P<0.001) (Fig. 2.5), suggesting that this primer pair displayed preferential amplification of certain AMF lineages.

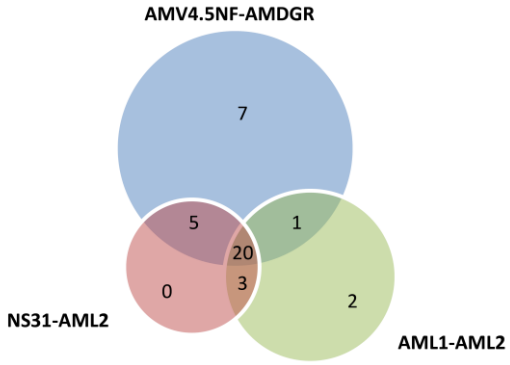


Figure 2.4 Venn diagram showing the number of shared and unique OTUs between the AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 primer pairs based on 8 samples (two replicates), an 190 bp overlapping SSU rRNA gene fragment and 350 AMF sequences per sample.

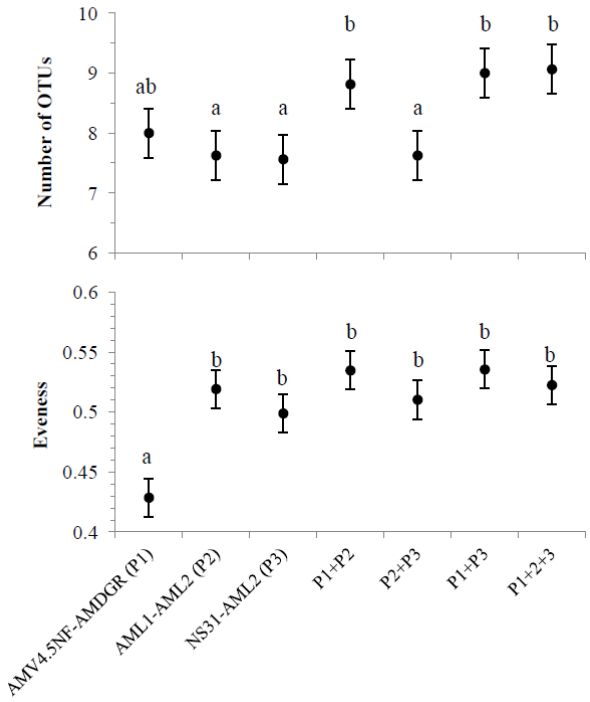


Figure 2.5 OTU richness and evenness of the AMF communities obtained with the primer pairs AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 based on 8 samples (two replicates), an 190 bp overlapping SSU rRNA gene fragment and 350 AMF sequences per sample. Additionally, number of OTUs and evenness are also shown for combinations of the primer pairs (also rarified to 350 AMF sequences per sample). Different letters represent significant differences at P=0.05 and error bars indicate standard errors.

In order to assess potential bias towards specific AMF families, the relative abundance of the different AMF families was compared between the different primer pairs (Table 2.5). In general, the majority of the AMF sequences belonged to the Claroideoglomeraceae (39.93-44.04%), Gigasporaceae (12.34-12.43%) and Glomeraceae (34.95-47.39%), whereas the Ambisporaceae, Diversisporaceae and Paraglomeraceae together accounted for less than 11% of the sequences. The AMV4.5NF-AMDGR primer pair yielded significantly more Glomeraceae sequences in comparison with the other primer pairs. This was also the case for the AMV4.5NF-AMDGR + AML1-AML2 and AMV4.5NF-AMDGR + NS31-AML2 primer pair combinations. Ambisporaceae were not detected

using AMV4.5NF-AMDGR, while the other primer pairs generated at least a few Ambisporaceae sequences. Additionally, significantly fewer sequences originating from the families Claroideoglomeraceae, Diversisporaceae and Paraglomeraceae were obtained with AMV4.5NF-AMDGR. Particularly for the Paraglomeraceae, a large difference was found between AMV4.5NF-AMDGR (0.11%) and other primer pairs (8.84-10.07%). Only minor differences in the abundance at the family level were found between the AML1-AML2 and NS31-AML2 primer pairs, an observation which was also mirrored in the combined analysis of these primer pairs. When combining results from the AMV4.5NF-AMDGR primer pair with AML1-AML2 and NS31-AML2, the influence of the AMV4.5NF-AMDGR primer pair is primarily be observed in a lower proportion of Paraglomeraceae and a higher proportion of Glomeraceae sequences. Finally, only minor differences could be observed between the AMV4.5NF-AMDGR + AML1-AML2 primer pair combination, the AMV4.5NF-AMDGR + NS31-AML2 primer pair combination and the combination of all three primer pairs (Table 2.5).

Table 2.5 Average proportion (%) of AMF sequences per sample based on 8 samples (2 replicates), an 190 bp fragment of the SSU rRNA gene and 350 AMF sequences per sample[a].

	AMV4.5NF-AMDGR (P1)	AML1-AML2 (P2)	NS31-AML2 (P3)	P1+P2	P2+P3	P1+P3	P1+2+3
Ambisporaceae	0.00 a	0.13 b	0.07 ab	0.11 ab	0.13 b	0.04 ab	0.04 ab
Claroideoglomeraceae	39.93 a	43.09 bc	44.04 c	41.73 b	43.27 bc	41.75 b	42.00 b
Diversisporacea	0.16 a	0.63 bc	0.52 abc	0.34 ab	0.71 c	0.39 abc	0.48 abc
Gigasporaceae	12.41 a	12.38 a	12.43 a	12.34 a	12.38 a	12.41 a	12.43 a
Glomeraceae	47.39 a	34.95 b	32.88 b	40.80 c	33.77 b	40.21 c	38.82 c
Paraglomeraceae	0.11 a	8.84 be	10.07 b	4.68 cd	9.75 b	5.20 de	6.23 bcd
	100%	100%	100%	100%	100%	100%	100%

[a]Different letters represent significant differences between primer pairs at P=0.05.

2.5 Discussion

Here, we evaluated the performance of six previously developed PCR primer pairs (NS31-AM1, AMV4.5NF-AMDGR, AML1-AML2, NS31-AML2, FLR3-LSUmBr and Glo454-NDL22) for characterization of AMF communities using 454 pyrosequencing, based on both an *in silico* primer analysis and 454 pyrosequencing of AMF communities from apple tree roots. In depth *in silico* analysis revealed differences in the ability of the different primers to detect AMF. More specifically, whereas the SSU targeting primers NS31, AMDGR and AML2 and the LSU targeting primer NDL22, showed perfect matches to almost all Glomeromycota sequences screened, less perfect matches (often depending on the AMF family) were obtained for the other primers tested, indicating the importance of careful primer selection. From all combinations, NS31-AML2 was the only pair that almost perfectly matched all AMF sequences.

Further evaluation of the six selected primer pairs by 454 pyrosequencing of the AMF communities from 18 apple tree root samples revealed that three primer pairs had broad fungal detection

capabilities, while the other three showed higher specificity towards Glomeromycota. More specifically, for NS31-AM1, FLR3-LSUmBr and Glo454-NDL22, almost no Glomeromycota sequences were obtained from the different samples (0.95%, 3.36% and 0.33%, respectively). In contrast to our findings, Öpik *et al.* (2009) and Dumbrell *et al.* (2011) found an AMF specificity of >70% with the NS31-AM1 primer pair. Additionally, Lekberg *et al.* (2012) obtained an AMF specificity of 41.4% with the Glo454-NDL22 primers. These discrepancies can be explained by the base composition of the primers in combination with the AMF abundance in the samples. Whereas NS31 and NDL22 were designed as universal eukaryotic primers (Simon *et al.*, 1992; van Tuinen *et al.*, 1998), also FLR3 and Glo454 were found to perfectly match a large number of Basidiomycota sequences (Krüger *et al.*, 2009). Additionally, the 3' terminal region of the AMF 'specific' primer AM1 was found to match with numerous non-AMF sequences (Lee *et al.*, 2008). Altogether, this may explain the high number of non-AMF sequences detected in this study using these three primer sets. Although these three primer pairs were successfully used in diverse AMF studies previously (Öpik *et al.*, 2009; Lekberg *et al.*, 2012), our results caution against the usage of these primer pairs without a preliminary evaluation of their performance in the system under investigation. In contrast, the majority of the sequences obtained with the SSU-targeting primer pairs AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 were Glomeromycota sequences. The AMF specificity of AMV4.5NF-AMDGR (72%) matches very well with the findings of Lumini *et al.* (2010), who found a specificity of 76%. AML1-AML2, consisting of two primers particularly designed for AMF specificity (Lee *et al.*, 2008), was the most specific primer pair tested as all sequences we obtained (100%) belonged to the Glomeromycota. However, it has to be noted that AML1 was found to have 1 mismatch in the 3' end primer region when compared to Ambisporaceae sequences (16.2% of the sequences screened), and therefore Ambisporaceae sequences may be underrepresented. While primer pair NS31-AML2 was found to be the most promising combination in the *in silico* primer analysis, combining the AML2 reverse primer with the universal forward primer NS31, reduced AMF specificity to 77%.

The proportion of high-quality sequences (truncated to 200-220 bp) obtained per primer pair varied considerably. The AMV4.5NF-AMDGR primer pair resulted in 61.3% high-quality reads, while this was 46.4% for AML1-AML2 and only 36.1% for NS31-AML2. It is unclear whether this difference was, due to PCR errors or 454 pyrosequencing-related limitations, for example. Additionally, for three samples no PCR product was obtained using primer pairs AML1-AML2 and NS31-AML2, whereas AMV4.5NF-AMDGR yielded on average 2260 AMF sequences per sample for these samples. When excluding these samples from the analysis, however, no significant difference in the number of high-quality sequences retained per sample could be found between the primer pairs.

Focusing on the primer pairs with high AMF specificity (AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2) revealed highly similar results for both replicates, indicating a high reproducibility of the PCRs to characterize AMF communities. No differences were found in OTU richness between the three primer pairs when evaluating the overlapping sequences generated by the three primer pairs (190 bp segment). Evenness, on the other hand, was lower for AMV4.5NF-AMDGR, indicating this primer pair preferentially amplified or missed certain AMF lineages and significantly more

Glomeraceae sequences were obtained with AMV4.5NF-AMDGR in comparison with the other two primer pairs. Additionally, no Ambisporaceae sequences and fewer sequences from Claroideoglomeraceae and Paraglomeraceae were obtained using AMV4.5NF-AMDGR. This is in line with results from the *in silico* primer analysis, where AMV4.5NF was found to have some mismatches, especially with sequences from Claroideoglomeraceae and Gigasporaceae. When combining results from the primer pairs AMV4.5NF-AMDGR and AML1-AML2 or AMV4.5NF-AMDGR and NS31-AML2, a higher number of OTUs per sample was found, illustrating the complementary character of both primer pairs. Combining all three primer pairs did not result in additional information, as results obtained for AML1-AML2 and NS31-AML2 were highly similar.

To conclude, we have shown that 454 pyrosequencing is a powerful approach to characterize AMF communities, but that different primer pairs may lead to different results, illustrating that comparison of studies using different primer pairs is difficult. Our results indicate that the AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 primer pairs are powerful primer pairs for the characterization of AMF communities. They have few mismatches to AMF sequences, a high selectivity towards Glomeromycota, and are able to consistently describe AMF communities. However, our results also suggest that Glomeraceae sequences are favored with the AMV4.5NF-AMDGR primer pair at the expense of Ambisporaceae, Claroideoglomeraceae and Paraglomeraceae sequences. Additionally, our results indicate that a combination of AMV4.5NF-AMDGR and AML1-AML2 or AMV4.5NF-AMDGR and NS31-AML2 may present a powerful complementary combination for tandem use when studying AMF communities.

Chapter 3

Decrease in diversity and changes in community composition of arbuscular mycorrhizal fungi in roots of apple trees with increasing orchard fertilization

Adapted from:

Van Geel Maarten, Ceustermans An, Van Hemelrijck Wendy, Lievens Bart, Honnay Olivier. 2015. Decrease in diversity and changes in community composition of arbuscular mycorrhizal fungi in roots of apple trees with increasing orchard management intensity across a regional scale. *Molecular Ecology*, **24**: 941-952.

3.1 Summary

Understanding which factors drive the diversity and community composition of arbuscular mycorrhizal fungi (AMF) is important due to the role of these soil microorganisms in ecosystem functioning and current environmental threats to AMF biodiversity. Additionally, in agro-ecosystems, this knowledge may help to evaluate their use in making agriculture more sustainable. Here, we used 454-pyrosequencing of small subunit rRNA gene amplicons to quantify AMF diversity and community composition in the roots of cultivated apple trees across 24 orchards in central Belgium. We aimed at identifying the factors (soil chemical variables, organic *versus* conventional farming, and geographical location) that affect AMF diversity and community composition. In total, 110 AMF OTUs were detected, of which the majority belonged to the Glomeraceae (73%) and the Claroideoglomeraceae (19%). We show that soil characteristics and farming system, rather than the geographical location of the orchards, shape AMF communities on apple trees. Particularly, plant-available P content of the soil was associated with lower AMF diversity. In orchards with a lower plant-available P content of the soil (P < 100 mg/kg soil), we also found a significantly higher AMF diversity in organically managed orchards as compared to conventionally managed orchards. Finally, the degree of nestedness of the AMF communities was related to plant-available P and N content of the soil, pointing at a progressive loss of AMF taxa with increasing fertilization. Overall, we conclude that a combination of organic orchard management and moderate fertilization may preserve diverse AMF communities on apple trees, and that AMF in the roots of apple trees appear not to be dispersal limited at the scale of central Belgium.

3.2 Introduction

Arbuscular mycorrhizal fungi (AMF, Glomeromycota) are an ubiquitous but rather species-poor group of obligate plant symbionts which are of particular value for the functioning of natural and agricultural ecosystems (Verbruggen & Kiers, 2010). AMF associate with roots of the majority of land plants and act as a living interface between plant roots and the soil (Smith & Read, 2008). Using their large extraradical mycelia network, AMF improve plant uptake of inorganic phosphorus (P) (Lekberg & Koide, 2005) and nitrogen (N) (Leigh *et al.*, 2009), in exchange for plant assimilated carbohydrates. In addition, AMF enhance formation of water-stable soil aggregates through the action of mycelium and glomalin-related soil proteins, and as a consequence also improve soil moisture, soil structure and drought tolerance of the host plant (Augé *et al.*, 2001; Wilson *et al.*, 2009). Finally, AMF may protect their hosts against fungal and nematode pathogens (Sikes *et al.*, 2009; Veresoglou & Rillig, 2012).

Previous studies have shown that there is considerable functional diversity in AMF, even within a species, affecting various important functions such as colonization rates, growth of extra-radical hyphae, and P uptake (Hart & Reader, 2002; Munkvold *et al.*, 2004; Jansa *et al.*, 2005; Angelard *et al.*, 2010). Because of their potential functional complementarity, it is expected that high AMF diversity is more beneficial for host plants than low diversity (van der Heijden *et al.*, 1998; Johnson *et al.*, 2003; Maherali & Klironomos, 2007). However, the diversity of AMF is known to decline following anthropogenic disturbances such as excessive use of chemical fertilizers and pesticides, and many valuable ecotypes could become extinct before they are even discovered (Turrini & Giovannetti, 2012). Given the known beneficial effects of AMF in general, their particular value for the sustainable functioning of agricultural ecosystems, and the current environmental threats to AMF diversity, it is crucial to identify the major environmental drivers of AMF communities in agricultural settings.

Generally, there is a broad agreement that soil characteristics strongly affect AMF communities in agricultural soils (Verbruggen *et al.*, 2012; Hazard *et al.*, 2013; Jansa *et al.*, 2014). Especially the plant-available P content of the soil has been recognized as one of the primary factors affecting AMF abundance. Increased levels of inorganic P inhibit the colonization of roots by various AMF taxa (Kahiluoto *et al.*, 2001; Jansa *et al.*, 2009). Also the type of agricultural management can have a strong effect on AMF. Compared to conventional farming, organic farming, which typically excludes chemical fertilizers, herbicides and pesticides, has already been shown to have a positive effect on the diversity of a wide range of taxa, including birds, mammals, invertebrates and plants (Hole *et al.*, 2005; Tuck *et al.*, 2014). Evidence that organic farming also conserves or promotes AMF diversity is more scarce so far, and is mostly based on field scale investigations using trap cultures and microscopic analysis of spores, which likely do not provide full insight in true mycorrhizal diversity (e.g. Oehl *et al.*, 2004; Purin *et al.*, 2006; Bedini *et al.*, 2013). To our knowledge, only four studies so far have used molecular techniques to study AMF diversity in organic management agricultural systems (Galvan *et al.*, 2009; Verbruggen *et al.*, 2010; Verbruggen *et al.*, 2012; Jansa *et*

al., 2014). However, these studies used real-time PCR and terminal restriction fragment length polymorphism (T-RFLP) analysis and thus likely lacked sufficient resolution to thoroughly characterize AMF diversity. In contrast, next generation sequencing (NGS) technologies, such as 454 pyrosequencing (Margulies *et al.*, 2005), now enable highly efficient characterization of AMF communities (Öpik *et al.*, 2009). Furthermore, it remains unclear whether organic farming may increase AMF diversity through minimizing impacts on soil characteristics such as the degree of fertilization, or through the prohibition of the use of herbicides and pesticides.

Most studies do not account for geographical variation when quantifying AMF communities in agricultural settings. However, geographical distance has been demonstrated to affect AMF communities, suggesting that dispersal limitation can be an important determinant of the occurrence of AMF taxa (Jansa *et al.*, 2014). On the other hand, Hazard *et al.* (2013) showed that AMF taxa across Ireland are not dispersal limited but that it is rather the local environment that acts as a filter.

In this study, we focus on the AMF associated with the roots of cultivated apple trees. Although microscopic studies have shown before that apple rootstock can host a relatively high AMF diversity (Miller *et al.*, 1985; Cavallazzi *et al.*, 2007), little is known regarding the specific AMF associated with cultivated apple trees, and regarding the response of these AMF to environmental variation across orchards. Therefore, the first objective of this study was to identify the AMF occurring in the roots of cultivated apple trees in fruit production areas in Central Belgium. Second, we aimed at determining which factors (soil chemical variables, management type (conventional vs. organic) and geography) drive AMF richness, diversity and community composition. To accomplish this, we utilized 454 pyrosequencing on root samples of 120 apple trees from 24 apple orchards, strongly different in degree of soil fertility, among which 18 conventionally and 6 organically managed orchards.

3.3 Materials and methods

3.3.1 Study sites and sampling

The study was conducted in the South of Flanders, the central part of Belgium (Appendix Fig. B1). This region with loamy and sandy loam soils has a maritime mesothermic climate with significant precipitation in all seasons. Annual average precipitation is 785 mm and average annual temperature is 9.8°C (Royal Meteorological Institute, Ukkel, Belgium). A total of 24 orchards (average distance between orchards was 28.5 km, minimal 2 km, maximal 100 km) (Appendix Fig. B1 and Table B1) were examined within this study. We sampled 18 conventionally managed orchards, including orchards with either a known low or a high degree of soil fertility, and we also included 6 organic orchards. The relative size (highest value divided through the lowest value) of the gradient was 1.59 for pH (logarithmic scale), 1.8 for organic carbon content, 5.98 for Olsen P and 36.91 for Soil N. In the organic orchards, no chemical fertilizers, herbicides and pesticides were used since at least 15 years. However, organic fertilizers and small amounts of copper-based fungicides (maximum 3 kg/hectare/year) to control apple scab have been applied at regular time

intervals. Furthermore, whereas in the conventional orchards a wide range of herbicides has been applied to keep the area at the base of the trees void of vegetation, in the organic orchards this is achieved through mechanical removing of weeds (c. 5 times per year). There were no differences in planting density or tree age between both types of orchards. All apple trees were of the 'Jonagold' cultivar and grafted on M9 rootstocks, the most commonly used rootstock for commercial apple production in Belgium. In August 2012, roots from five randomly chosen apple trees per orchard were excavated. Root samples were collected on three random locations of the root system and were pooled afterwards to obtain one pooled root sample per tree. Consequently, in total 120 apple trees were sampled. Especially fine roots were collected, as these are known to contain AMF (Guo *et al.*, 2008). Root samples were stored at 4 °C until further use. In parallel, 25 soil cores were randomly taken in each orchard for chemical analysis. These cores were pooled and mixed to obtain one soil sample of approximately five liter per orchard. Soil samples were stored at 4 °C until further analysis.

3.3.2 Soil chemical analysis

Soil pH was determined using a pH probe in a 1:10 soil/water mixture. As a measure of the plant-available N content of the soil, ammonium and nitrate availability was determined by shaking 10 g of soil in 200 mL of 1 M potassium chloride solution for one hour. Extracts were analyzed colorimetrically using a segmented flow auto analyzer (Skalar, Breda, the Netherlands). As a measure of the plant-available P content of the soil, Olsen P values were determined by shaking 2 g dry soil for 30 minutes with 0.5 M sodium bicarbonate at pH 8.5 and subsequent colorimetric analysis of the extracts using the molybdenum blue method (Robertson *et al.*, 1999). Organic carbon content was determined by shaking 10 g of soil in an excess volume of 0.27 M potassium dichromate and 18 M sulfuric acid at a temperature of 135 °C. Extracts were analyzed colorimetrically.

3.3.3 DNA extraction, PCR amplification and pyrosequencing

Following microscopic verification of the presence of mycorrhizal fungi, obtained roots samples were cut in 1 cm pieces and rinsed twice with sterile distilled water. For each sample, 0.1 g root material was used to extract DNA using the UltraClean Plant DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer's instructions. Subsequently, the obtained DNA was diluted 10 times prior to PCR amplification. PCR amplification was performed using primer pair AMV4.5NF-AMDGR (Sato *et al.*, 2005), as this primer pair is highly AMF specific and is able to consistently describe AMF communities using 454 pyrosequencing based on the most variable part of the small subunit (SSU) rRNA gene region (Van Geel *et al.*, 2014). 'Fusion' primers, required for the 454 process, were designed according to the guidelines for 454 GS-FLX Titanium Lib-L sequencing containing the Roche 454 pyrosequencing adapters and a sample-specific MID barcode in between the adapter and the forward primer. In total, 60 MID barcodes (recommended by Roche, Mannheim, Germany) were used for sample-specific amplicon tracking. PCR reactions were performed on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) in a reaction volume of 20 µl, containing 0.15 mM of each dNTP, 0.5 µM of each primer, 1x Titanium *Taq* PCR

buffer, 1U Titanium *Taq* DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), and 1 µl genomic DNA. Before amplification, DNA samples were denatured at 94°C for 2 min. Next, 35 cycles were run, consisting of 45 s at 94°C, 45 s at 65°C and 45 s at 72°C, followed by a final elongation of 10 min at 72°C. After resolving the amplicons by agarose gel electrophoresis, amplicons within the appropriate size range were cut from the gel and purified using the Qiaquick gel extraction kit (Qiagen, Hamburg, Germany). Purified dsDNA amplicons were quantified using the Quant-iT PicoGreen® dsDNA Assay Kit and the Qubit fluorometer (both from Invitrogen, Ghent, Belgium), and pooled in equimolar quantities over two amplicon libraries, each representing 60 samples tagged with a unique MID barcode. The quality of the amplicon libraries was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The amplicon libraries were each loaded on a 1/4th of a 454 Pico Titer Plate and pyrosequencing was performed using the Roche GS-FLX instrument and Titanium chemistry according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

3.3.4 Bioinformatics

Sequences obtained from the 454 pyrosequencing run were clustered into operational taxonomic units (OTUs) using the UPARSE algorithm, following the recommended pipeline (Edgar, 2013). First, quality filtering of the reads was performed with the 'fastq_filter' command, allowing a maximum expected error of 0.5 for the individual sequences. In order to maximize the number and length of retained sequences, truncation length was set to 225 bp (average length was 261 bp). Next, the sequences were dereplicated and sorted by abundance. Subsequently, singletons, i.e. sequences only occurring once in the entire dataset, were removed prior to clustering as this has been shown to improve the accuracy of diversity estimates (Kunin *et al.*, 2010; Tedersoo *et al.*, 2010a; Waud *et al.*, 2014). Then, sequences were clustered into OTUs defined at 97% sequence similarity, which is commonly used to define SSU-based OTUs in AMF (Lumini *et al.*, 2010; Öpik *et al.*, 2010), with the 'cluster_otus' command. In this step, chimeric OTUs built from more abundant reads were discarded as well. However, few chimeras may be missed if their parents were absent or present with very low abundance. Therefore, obtained OTUs were double-checked for chimeric sequences against the MaarjAM database (Öpik *et al.*, 2010) using the 'uchime_ref' command. OTUs were assigned to a taxonomic identity by querying the representative sequence (as determined by the 'cluster_otus' command) against GenBank using the BLAST algorithm (Altschul *et al.*, 1990). Taxonomic assignments were considered reliable when a ≥200 BLAST score value was found (Lumini *et al.*, 2010). OTUs not belonging to the Glomeromycota or having a BLAST score lower than 200 were discarded. To accurately identify the obtained AMF OTUs, the representative sequence for each OTU was also queried against the MaarjAM database (Öpik *et al.*, 2010; accessed 6 March 2014), a database that aims to provide a quality-controlled repository for published sequence data from Glomeromycota.

3.3.5 Data analysis and statistics

To assess the sampling effort, rarefaction curves were calculated in MOTHUR (Schloss *et al.*, 2009) for all 24 orchards, using a re-sampling without replacement approach. To prevent bias due to

different sequencing depth, all samples were subsequently rarefied to 1371 AMF sequences per sample (25th percentile). Samples with less than 1371 AMF sequences per sample were omitted from further analyses. AMF richness was determined as the number of AMF OTUs present in a sample. AMF diversity was approximated by the Shannon diversity index (H) and was calculated using EstimateS (Colwell *et al.*, 2012). Shannon diversity was exponentially transformed (Exp(H)). After this conversion, AMF diversity is measured in units of number of OTUs and the variable behaves linear, in contrast to the non-transformed Shannon diversity (see Jost (2006) for details). Richness and Exp(H) of each orchard was determined as the average of the richness and Exp(H) of the five apple tree root samples, respectively.

The relationships between richness, Exp(H) and soil chemical variables (pH, P, N and Organic carbon) were first explored by calculating Pearson correlation coefficients among each pair of variables. Richness and Exp(H) between organic and conventional orchards were compared using *t*-tests. To estimate the combined variance of richness and Exp(H) explained by soil chemical variables and management type (organic vs. conventional), multiple linear regressions with forward selection were performed in SPSS 20.0 (SPSS Inc., Chicago, IL).

To test for relationships between AMF community composition (i.e. presence/absence of certain OTUs in the AMF community), soil chemical variables, management type and geographical distance, we used unconstrained and constrained ordination. First, a set of spatial predictors were calculated from the geographical coordinates of the orchards by principle coordinates of neighbor matrices (PCNM), using the 'pcnm' function of the R-package Vegan (Borcard & Legendre, 2002; Borcard *et al.*, 2004; Oksanen *et al.*, 2013). For the unconstrained ordination approach, we performed a non-metrical multidimensional scaling (NMDS) on the orchard * OTU matrix, using Bray-Curtis distances based on presence/absence data (R- package Vegan, Oksanen *et al.*, 2013). Subsequently, soil chemical variables, management type and PCNM were fitted onto the ordination and tested for significance based on a permutation test with 1000 iterations, using the function 'envfit' (Vegan package). For the constrained ordination approach, canonical redundancy analysis (RDA) was performed using the R-package Vegan with the soil chemical variables, management type and PCNM as explanatory variables (Oksanen *et al.*, 2013). Note that RDA not only uses the presence/absence data, but also models the abundance of the OTUs. To determine the explanatory variables that significantly explained variation in the AMF communities, forward selection (999 Monte Carlo permutations, $\alpha < 0.05$) was used (R package Packfor). Variation partitioning was performed as proposed by Legendre (2008) using the 'varpart' function of the R-package Vegan. Only the significant explanatory variables, as determined by forward selection, in each of the three groups of predictors (soil chemical variables, management type and geography) were included in this analysis. Additionally, to test explicitly for the effect of geographic distances on the AMF communities, the correlation between Bray-Curtis AMF community and geographical distances between orchards was tested using a partial Mantel test in PC-ORD 6 (McCune & Mefford, 2006). Partial Mantel tests were used to be able to control for differences in soil chemical characteristics between orchards.

Finally, to test whether AMF communities showed a nested structure, i.e. to test whether AMF OTUs detected in OTU-poor orchards are a subset of the OTUs found in OTU-rich orchards, two different measures were used to estimate the degree of nestedness. First, a formal nestedness analysis was performed using BINMATNEST (Rodriguez-Girones & Santamaria, 2006). The program calculates the matrix temperature, a measure of nestedness varying between 0° (perfectly nested) and 100° (perfectly non-nested). The significance of nestedness was tested using default input parameters and null model 3. Almeida-Neto *et al.* (2008) demonstrated that matrix temperature may be sensitive to both matrix size and shape, and designed a new metric for nestedness analysis to overcome these flaws. This metric is based on overlap and decreasing fill (NODF) and was calculated using the software package ANINHADO (Guimarães & Guimarães, 2006). To test the significance of nestedness, two different randomization models were used. In the first model (ER) presences are randomly assigned to any cell within the matrix. In the second model (CE) the probability of each cell being occupied depends on the number of presences in the row and column (Almeida-Neto *et al.*, 2008). The CE model allows us to test for statistical significance, given that some orchards have higher diversity and some taxa are more common than others. In order to assess the relation between the nestedness of the AMF communities and orchard fertilization, a Spearman rank correlation coefficient was calculated between temperature and the soil chemical variables and a Mann-Whitney U test was performed to test for a significant difference in temperature between both management types.

3.4 Results

3.4.1 AMF diversity

454 pyrosequencing yielded a total of 438 194 sequences with a minimal length of 225 bp containing the correct barcode and primer sequence. A BLAST search against GenBank revealed the presence of 117 479 (24.6%) non-Glomeromycota sequences with the majority belonging to the Basidiomycota. After removal of the non-Glomeromycota sequences, 320 715 AMF sequences remained. The number of sequence reads per sample was strongly correlated with the number of OTUs per sample ($r = 0.70$, $P < 0.001$). Therefore, the number of sequences was rarefied to 1371 AMF sequences per sample, leaving 90 (still representing all 24 orchards sampled) out of the initial 120 samples for further analysis (Appendix Table B1).

In total, 110 AMF OTUs were detected in the 24 apple orchards (rarefied samples). The majority of OTUs belonged to the Glomeraceae (73%, 80 OTUs, 78394 sequences) and Claroideoglomeraceae (19%, 21 OTUs, 22713 sequences), whereas only few belonged to the Gigasporaceae (4%, 4 OTUs, 496 sequences), Diversisporaceae (3%, 3 OTUs, 176 sequences), Paraglomeraceae (1%, 1 OTU, 1964 sequences) and Acaulosporaceae (1%, 1 OTU, 38 sequences) (Appendix Table B2, rarefied samples). Most rarefaction curves tended to saturate at the chosen sequencing depth (5 times 1371 sequences per orchard) (Appendix Fig. B2), and richness ranged from 10 to 26 OTUs per orchard. The *t*-test showed both richness and Exp(H) to be significantly higher in the organic orchards compared to the conventional orchards (Table 3.1). Olsen P was strongly inversely correlated with

richness and Exp(H) (Table 3.2). Soil N was inversely correlated with richness, but not with Exp(H). No significant correlations were found between both AMF diversity measures and soil pH or organic carbon content. Multiple linear regression analyses with forward selection (F-to-enter at $P = 0.10$) revealed Olsen P ($P = 0.003$) as the sole significant explanatory variable for richness. This variable explained 34% of the richness (Table 3.3). The forward selection procedure for Exp(H) yielded Olsen P ($P = 0.016$) and management type ($P = 0.084$) as explanatory variables. Both variables together explained 41% of the Exp(H) across orchards (Table 3.3, Fig. 3.1). In order to disentangle the effects of Olsen P and management type, we additionally tested for an effect of management type in the orchards with Olsen P < 100 mg/kg only, using a t-test (Fig. 3.1). In these relatively P-poor orchards, a higher Exp(H) was observed in the organic orchards as compared to the conventional orchards ($t = -2.364$, $P = 0.032$), whereas richness did not differ significantly between management types ($t = -1.383$, $P = 0.185$).

Table 3.1 Results of the *t*-test on AMF richness and Shannon diversity (H) in roots of apple trees of 6 organic and 18 conventional orchards.

	Management	*N*	Mean	*t*	*P*
AMF richness	Organic	6	22.33	2.10	0.047
	Conventional	18	18.90		
Exp(H)	Organic	6	5.76	3.11	0.008
	Conventional	18	4.51		

Table 3.2 Pearson correlation coefficients between AMF richness and diversity, and soil chemical variables ($N = 24$, $*P < 0.05$, $**P < 0.01$).

	Richness	Exp(H)	pH	Olsen P	Soil N	Organic carbon
Richness	1	0.49*	0.13	-0.58**	-0.18	0.33
Exp(H)		1	0.05	-0.57**	-0.15	0.33
pH			1	-0.32	0.29	0.06
Olsen P				1	0.26	-0.41*
Soil N					1	-0.07
Organic carbon						1

Table 3.3 Multiple linear regression coefficients and their statistical significant difference from zero after model optimization, using forward selection. The soil variables pH, N and organic carbon were finally excluded by both forward selection model optimization procedures. Management was coded as a dummy variable (0 = Organic, 1 = Conventional) and finally excluded by the forward selection procedure in the analysis of AMF richness.

	Richness ($F_{1,24} = 11.2$, $P = 0.003$, $R^2 = 0.34$)			Exp(H) ($F_{2,24} = 7.4$, $P = 0.004$, $R^2 = 0.41$)		
	Coefficient	*t*	*P*	Coefficient	*t*	*P*
Intercept	23.999	16.93	<0.001	6.527	13.53	<0.001
Olsen P	-0.047	-3.35	0.003	-0.012	-2.627	0.016
Management	-	-	-	-0.847	-1.813	0.084

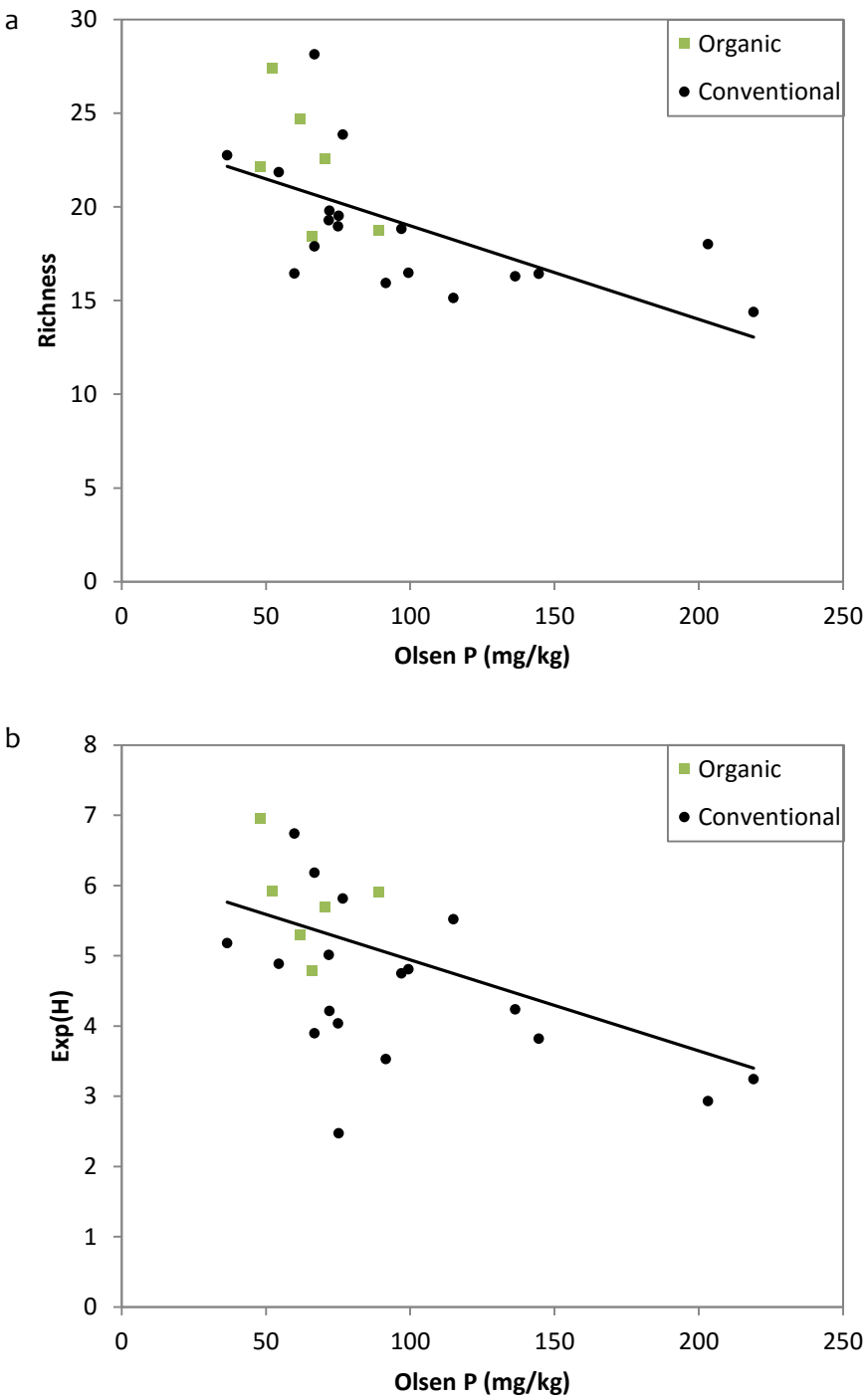


Figure 3.1 Relationship between AMF diversity measures (Richness (a), Exp(H) (b)) and soil Olsen P. Lines represent significant linear correlations at $P < 0.001$. Squares represent organically managed orchards; circles represent conventionally managed orchards.

3.4.2 Effect of management, soil chemical variables and geography on AMF community composition

The NMDS permutation test revealed organic orchards to harbor significantly different AMF communities as compared to conventional orchards (Table 3.4, Fig. 3.2). From the soil chemical variables, only Olsen P contributed significantly to AMF community composition (Table 3.4). No significant relations could be found between AMF community composition and the six PCNM variables (Table 3.4).

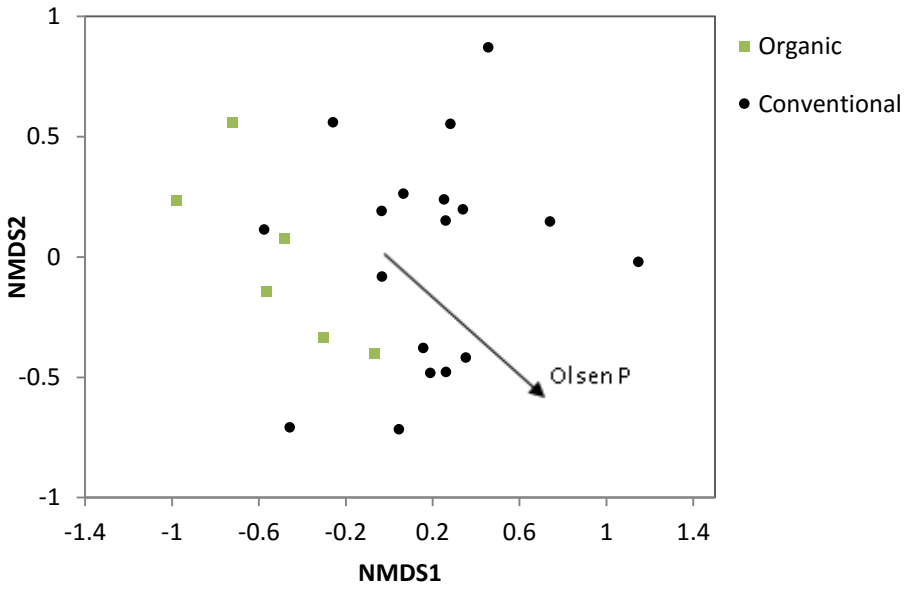


Figure 3.2 Non-metric multidimensional scaling ordination plot of arbuscular mycorrhizal fungal communities from 24 orchards. AMF communities between organic (squares) and conventional (circles) orchards were found to be significantly different (Table 3.4). Only the significant relationships between ordination scores and environmental variables are shown with an arrow, representing the direction of the increasing gradient. Stress value: 17.2.

Table 3.4 Results of the permutation tests of the two dimensional non-metric multidimensional scaling coordinates testing for significant relationships between AMF community composition and management, soil chemical variables and PCNM (spatial predictors). The results are based on 1000 permutations.

	R^2	*P*
Management	0.404	0.002
pH	0.203	0.100
Olsen P	0.366	0.013
Soil N	0.221	0.068
Organic carbon	0.190	0.106
PCNM1	0.075	0.434
PCNM2	0.216	0.093
PCNM3	0.069	0.476
PCNM4	0.001	0.989
PCNM5	0.032	0.724
PCNM6	0.101	0.342

In agreement with the NMDS permutation test, the forward selection procedure in the direct ordination approach selected Management (R^2_a = 0.054, *P* = 0.009) and Olsen P (R^2_a = 0.047, *P* =

0.017) as the only explanatory variables. Together these variables explained 10.1% of the variation in the AMF communities. Variation partitioning showed that 3.43% and 4.18% of the variation could be assigned to management type and soil chemical variables, respectively, while 2.53% of the explained variation could not be separated between both groups of predictors (Table 3.5). The six PCNM (spatial predictors) variables could not explain any significant part of variation in the AMF communities. Likewise, the partial Mantel tests showed no correlation between pairwise Bray-Curtis AMF community distances and geographical distances, while controlling for the soil chemical variables (Table 3.6).

Table 3.5 Canonical redundancy analysis for determination of percent variation of AMF communities. The six PCNM variables (spatial predictors) did not significantly explain any variation of AMF communities.

	% of variation
Management	3.43%
Soil	4.18%
Management&Soil	2.53%
Undetermined	90.36%

Table 3.6 Results of the partial Mantel tests for the relationship between geographic distances and AMF communities.

Parameter	Control for	r	*P*
Distance	Management	-0.053	0.690
Distance	pH	0.023	0.427
Distance	Olsen P	0.009	0.482
Distance	Soil N	0.009	0.474
Distance	Organic carbon	0.012	0.461

3.4.3 Nestedness of AMF communities

AMF communities across orchards were significantly nested. The matrix temperature of the total AMF community was 30.8° and was significantly lower than expected by chance (expected T = 54.6°, SD = 5.83, $P < 0.001$). In agreement, the matrix NODF(Er) was 33.72 ($P < 0.001$) and NODF(Ce) was 40.61 ($P < 0.001$), indicating that the matrix was significantly more nested than expected by chance. The row and column permutated presence/absence matrix closest to perfect nestedness is shown in Fig. 3.3, together with the temperature, a measure of nestedness, for each orchard. The orchards with the three highest temperature values, and therefore with the lowest degrees of nestedness, were all organic orchards. Nevertheless, a Mann-Whitney U test revealed no significant difference in 'temperature' between organic and conventional orchards ($Z = 1.67$, $P = 0.25$). In contrast, a significant correlation between 'temperature' and Olsen P (Spearman's rank, r = -0.52, $P = 0.009$) and Soil N (Spearman's rank, r = -0.46, $P = 0.025$) was found. Therefore, orchards having higher soil Olsen P and Soil N values had increasingly nested AMF communities.

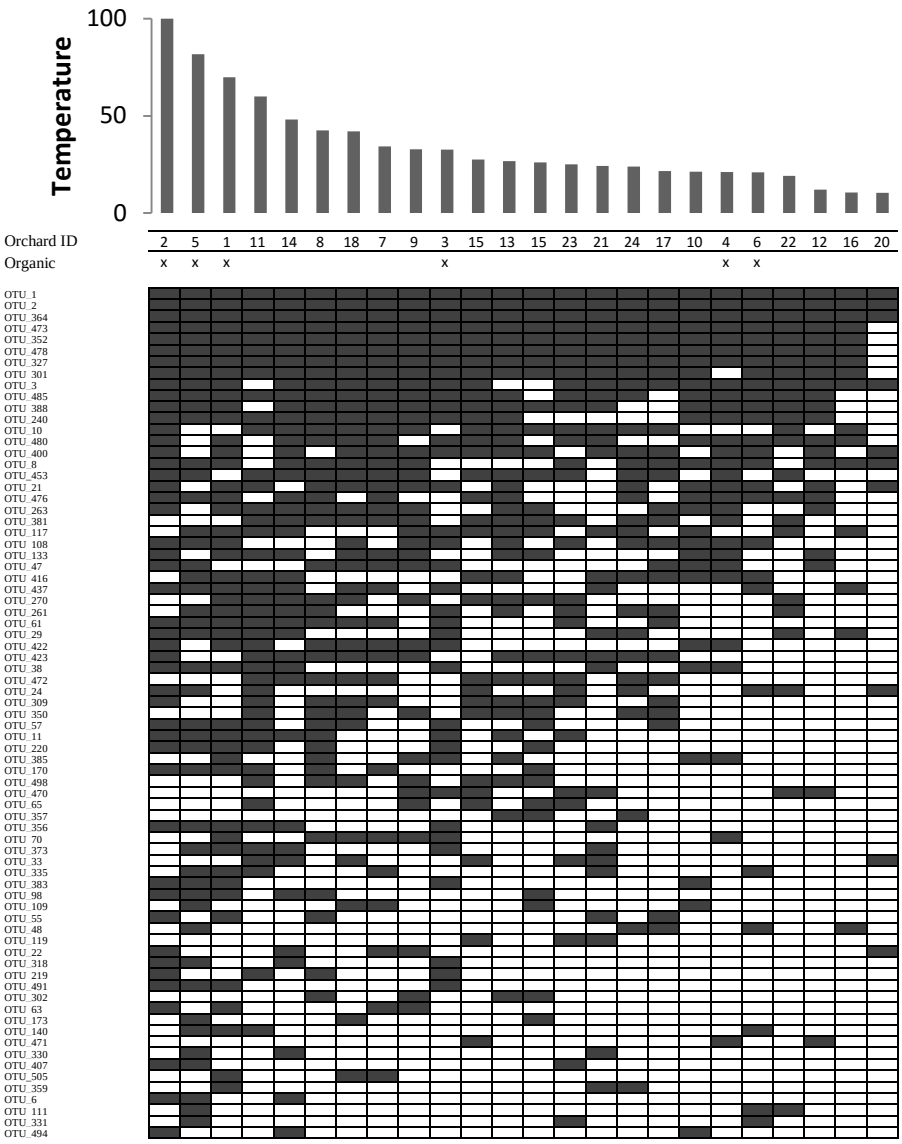


Figure 3.3 Nestedness of arbuscular mycorrhizal fungal communities from 24 orchards as shown by the row and column permutated presence/absence matrix that is closest to perfect nestedness. Columns represent orchards (sorted according to their degree of nestedness) and rows are OTUs. The 'temperature', a measure of nestedness, of each AMF community is shown on top of the matrix. Organic orchards are indicated with an X.

3.5 Discussion

3.5.1 AMF and soil chemical variables

There have been several studies on AMF diversity in agricultural settings, comparing organic and conventional management practices. However, the majority of these studies used trap cultures, microscopic analysis or molecular profiling techniques to identify AMF and thus lacked the power to thoroughly estimate AMF diversity (e.g. Verbruggen *et al.*, 2010; Bedini *et al.*, 2013). This is one of the first studies quantifying AMF richness, diversity and community composition in organically and conventionally managed agricultural soils across a regional scale using 454 pyrosequencing of SSU rRNA gene amplicons (but see Xiang *et al.*, 2014). It was found that the plant-available P content of

the soil (Olsen P) explained 34% of the richness and 32% of the Shannon diversity of AMF associated with apple roots in orchards, and was the only soil chemical variable that was related to AMF richness and diversity. No effects of the plant-available N content of the soil were found. These findings are in agreement with Verbruggen *et al.* (2012), who studied the AMF community composition of 40 agricultural soils in the Netherlands using T-RFLP analysis of large subunit (LSU) rRNA gene fragments. Lower AMF diversity under a high plant-available P content has been explained by both optimal resource allocation and biotic interactions (Johnson *et al.*, 2013). Optimal allocation predicts that plants should allocate biomass to structures that best garner the most limiting resources. Consequently, the enrichment by inorganic P through fertilization will reduce plant allocation to roots and the mycorrhizal symbiosis, therefore increasing the competition for carbohydrates between AMF species, potentially leading to a lower richness. Indeed, competition can be sufficiently strong to exclude some AMF species from host roots (Hepper *et al.*, 1988). Likewise, we also found very different AMF communities in orchards with a high and low plant-available P content of the soil.

The AMF communities detected across the sampled orchards were organized in a nested pattern. Poorer communities are therefore a subset of the richer communities, indicating a general trend of local taxa disappearance from the potential taxa pool. Through increased competition those AMF species that are most effective at acquiring plant carbohydrates under a high plant-available P content of the soil may have been favored. Therefore, sensitive AMF species may have disappeared along a fertilization gradient and cause community nestedness. Indeed, a relationship was observed between nestedness of the AMF communities and plant-available P and N content of the soil, suggesting that varying tolerance levels of AMF taxa are responsible for the observed nestedness. Similar observations have been made in the roots of maize and potato plants across 40 agricultural soils in the Netherlands (Verbruggen *et al.*, 2012).

3.5.2 AMF and management practices

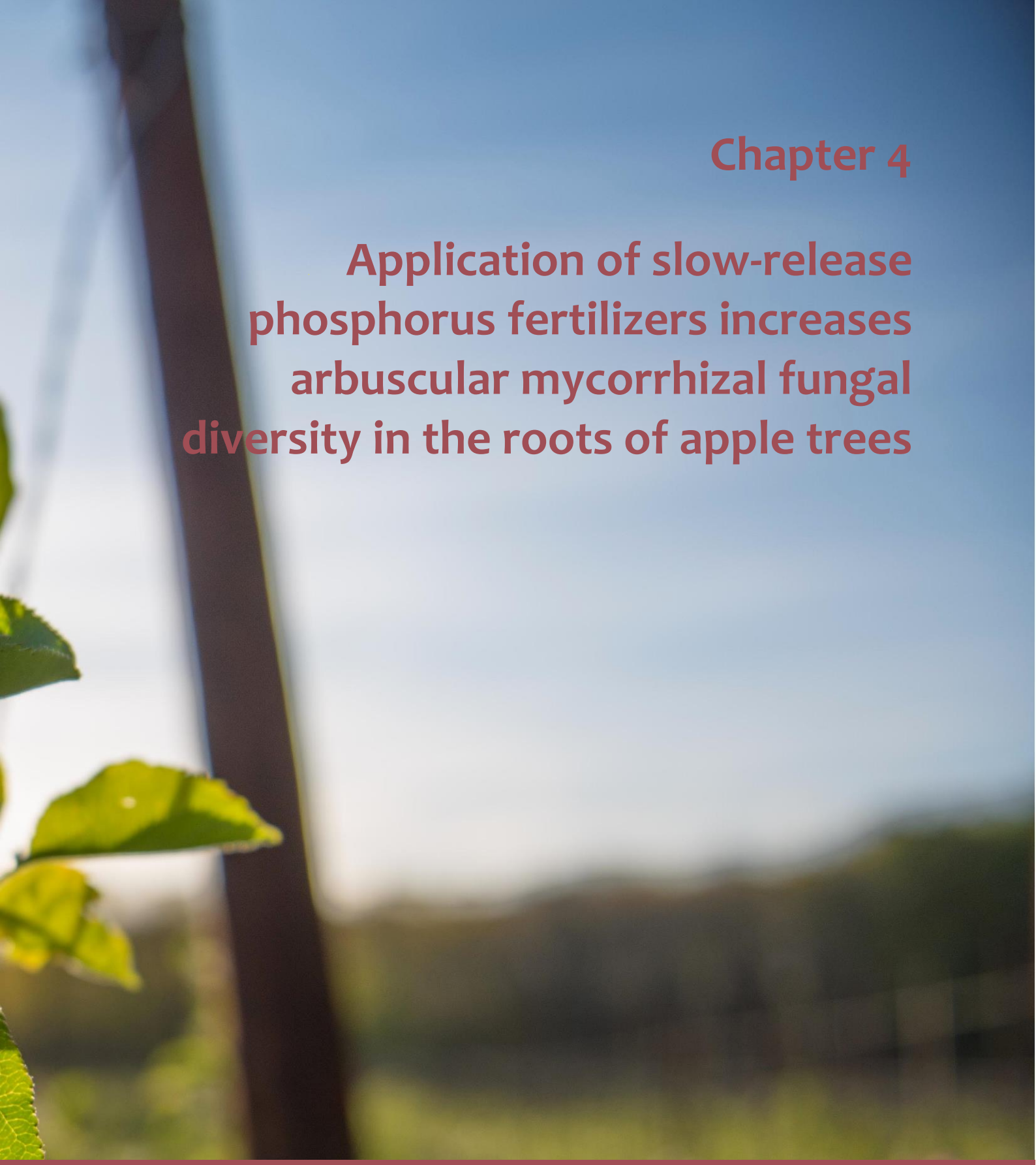
AMF community composition was found to differ between organic and conventional orchards, and we also found a marginally significantly positive effect of organic management on the Shannon diversity of AMF. As all organic orchards in this study had a soil plant-available P content lower than 100 mg/kg, this overall difference in AMF diversity and community composition may be partly explained by the lower soil fertility levels in organically management orchards. However, Shannon AMF diversity of conventional orchards with similar soil plant-available P content as the organic orchards (lower than 100 mg/kg), was significantly lower than Shannon AMF diversity of organic orchards. It is therefore not unlikely that also the exclusion of chemical biocides in organic management positively affects AMF diversity. It has indeed been demonstrated that chemical biocides can have a negative effect on AMF (Schreiner & Bethlenfalvay, 1996). Also the higher number of weeds present in organically managed orchards (Hole *et al.*, 2005) may explain the higher AMF diversity. Indeed, it is possible that changes in the AMF communities in the soil follow the changes in the weed communities (Johnson *et al.*, 2003; Zobel & Öpik, 2014). The question remains whether the higher AMF diversity present on the weeds may also increase the AMF

diversity on the apple roots (Brito *et al.*, 2013). A higher AMF diversity in organic orchards also corroborates previous observations that organic farming had a positive effect on the diversity of other microbial soil organisms, such as bacteria, other fungi and nematodes (Mäder *et al.*, 2002; Hole *et al.*, 2005). Based on spore analysis, Oehl *et al.* (2004) also found a higher AMF diversity in organically managed agricultural fields.

Given their role in soil fertility, plant nutrition and pathogen protection, AMF recently also received attention from a conservation point of view (Turrini & Giovannetti, 2012). On average, organic farming has been shown to increase species richness of a broad range of taxa by about 30% (Tuck *et al.*, 2014). Our results suggest that organic farming also contributes to AMF diversity, as we observed a 18% higher richness and a 28% higher Exp(H) diversity in organic as opposed to conventional orchards.

3.5.3 AMF and geography

No relationship between AMF communities and geographical distance were found, indicating that in our study geographical distance did not affect the distribution of AMF taxa. Hence, the AMF communities in our study were not dispersal limited. Additionally, no effect of the spatial (PCNM) variables was found on the AMF communities. Our analysis shows that AMF communities were mainly shaped by environmental variables. Similar results were obtained from 40 sites across Ireland, representing different land uses and soil types (Hazard *et al.*, 2013). These authors showed that AMF community composition in the roots of *Trifolium repens* and *Lolium perenne* was driven by the local environment, rather than by geographical distance. The scale (average distance between orchards was 28.5 km, minimum 2 km, maximum 100 km) of our study may explain the absence of a geographical distance effect. Environmental factors are often more important at local scales, while dispersal limitation may become more important at regional scales (250 km) (Martiny *et al.*, 2011). For example, it has been reported that AMF community composition in agricultural soils across England changed significantly across the regional scale (van der Gast *et al.*, 2011).



Chapter 4

Application of slow-release phosphorus fertilizers increases arbuscular mycorrhizal fungal diversity in the roots of apple trees

Adapted from:

Van **Geel Maarten, De Beenhouwer Matthias, Ceulemans Tobias, Caes Kenny, Ceustermans An, Bylemans Dany, Gomand An, Lievens Bart, Honnay Olivier.** 2016. Application of slow-release phosphorus fertilizers increases arbuscular mycorrhizal fungal diversity in the roots of apple trees. *Plant and Soil*, **402:** 291-301

4.1 Summary

Arbuscular mycorrhizal fungi (AMF) play a key role in the functioning of agricultural ecosystems. Therefore, understanding how the application of fertilizers, a common management practice, affects AMF communities is of major importance. Here we aimed to: (i) experimentally test whether different amounts and forms of phosphorus (P) fertilizer affect AMF diversity and community composition associated with the roots of apple trees (*Malus x domestica*); (ii) identify differences in tolerance to P fertilization between AMF taxa. We used 454-pyrosequencing of the small subunit rRNA gene amplicons to quantify AMF diversity and community composition in root samples obtained from a three year field experiment, with two inorganic, three slow-release P fertilization and one control treatment. The slow-release fertilizer treatments showed significantly higher AMF richness and differed in community composition compared to the inorganic fertilizer treatments. The distribution of AMF OTUs showed a significantly nested pattern. Additionally, AMF communities in the inorganic fertilizer treatments were a subset of the communities in the slow-release fertilizer treatments. We demonstrate that application of slow-release fertilizers promoted AMF diversity in the roots of cultivated apple trees in comparison to the other treatments. The application of inorganic fertilizers elevated levels of plant-available P in the soil and selected only a small subset of abundant AMF, resulting in a lower AMF diversity. This may result in AMF communities dominated by inferior AMF mutualists.

4.2 Introduction

Arbuscular mycorrhizal fungi (AMF, Glomeromycota) are widespread and obligate plant symbionts that form a symbiosis with the majority of land plants, including most agricultural crops (Smith & Read, 2008). They increase uptake of soil nutrients, especially phosphorus (P), and improve the stress tolerance of their hosts in return for plant photosynthates (Smith & Read, 2008). Furthermore, AMF improve soil formation and soil aggregation (Rillig & Mummey, 2006; Wilson *et al.*, 2009) and they provide protection of the host plant against fungal and nematode pathogens (Sikes *et al.*, 2009; Veresoglou & Rillig, 2012). The benefits of the AMF symbiosis have the potential to increase agricultural productivity in a sustainable way, as they decrease dependency on external inputs such as inorganic fertilizers and pesticides (Verbruggen & Kiers, 2010). Therefore, understanding how common agricultural management practices affect AMF communities is of major importance, as this may guide us to select those management types with the least impact on these beneficial fungi.

One of the most important agricultural management practices that is known to strongly affect AMF abundance, composition and diversity of crops is the application of soil fertilizers. Fertilization decreases limitation of belowground resources relative to aboveground resources and will thus increase resource allocation to shoots and leaves, and reduce root biomass and the degree to which they support AMF (Johnson, 2010; Johnson *et al.*, 2013). Many observational studies have shown the detrimental effects of fertilization on AMF communities. For example, it has been shown that the plant-available P in the soil was negatively correlated with the AMF diversity in the roots of meadow fescue and yarrow (Santos *et al.*, 2006), and maize and soybean (Gosling *et al.*, 2013). Moreover, Verbruggen *et al.* (2012), Van Geel *et al.* (2015) and De Beenhouwer *et al.* (2015) found that P availability in the soil explained most of the variation in AMF richness in the roots of maize and potato, apple trees and coffee shrubs, respectively. Overall, there is agreement that plant-available P is the most important soil characteristic affecting AMF communities.

Although the above mentioned observational studies have provided valuable insights into the relative importance of different environmental variables affecting AMF communities, only experimental approaches can establish causal relationships between AMF community composition and the application of P fertilizer. For example, Jensen and Jakobsen (1980) showed that a long-term P fertilizer application increased the plant-available P in the soil, and subsequently lowered AMF abundance. Furthermore, 8 years of inorganic N and P additions decreased the abundance of *Gigaspora gigantea*, *Gigaspora margarita*, *Scutellospora calospora* and *Paraglomus occultum* in the roots of big bluestem grass, whereas the abundance of *R. intraradices* increased (Johnson, 1993). Additionally, Kahiluoto *et al.* (2000) have shown that although both spore density and root colonization of flax, barley and red clover decreased after P fertilization, there were no changes in AMF community composition in the roots of the hosts. Far most experimental P fertilization studies relied on microscopic analysis of spores to identify AMF taxa, an approach which is known to be potentially flawed and which does not allow insight in the true mycorrhizal diversity (Sanders,

2004). More recently, approaches based on genetic finger printing and Sanger sequencing of cloned amplicons have been used, but these studies have suffered from a lack of resolution (Alguacil *et al.*, 2010; Chen *et al.*, 2014). The advent of next generation sequencing technologies, such as 454 pyrosequencing (Margulies *et al.*, 2005), have enabled the highly efficient characterization of AMF communities and have revealed unexpectedly high AMF diversities (Öpik *et al.*, 2009; Lumini *et al.*, 2010; Van Geel *et al.*, 2015). These findings may challenge the current knowledge of the response of AMF communities to experimental P fertilization. Lin *et al.* (2012), for example, used 454 pyrosequencing and found that AMF diversity in an arable soil in North China decreased after long-term balanced fertilization.

So far, very little is also known regarding the effects of different types of P fertilizer, including mineral, organic or compost, on the AMF communities in the roots of the host. AMF response may depend on the rate at which the fertilizer releases its components, which is known to vary for different types of P fertilizer. More specifically, it can be expected that P fertilizer types that slowly release their components may have less detrimental effects on the AMF communities, as compared to P fertilizer types that are readily available to the plant. Indeed, it has been shown that as long as plant-available P in the soil remains low, addition of P-rich compost has no detrimental effects on AMF colonization (Gaur *et al.*, 2000). Gryndler *et al.* (2006), reported that AMF colonization decreased after addition of inorganic P fertilizer, and that it increased after addition of organic P fertilizer. On the other hand, if organic P fertilizer results in rapid mineralization of P, it increases the plant-available P, resulting in a negative impact on the AMF community (Sainz *et al.*, 1998).

In this study, we investigated the effect of different P fertilizer types on the AMF communities in the roots of apple. Apple trees are known to harbor a high AMF diversity (Cavallazzi *et al.*, 2007; Van Geel *et al.*, 2015). Our specific objectives were to: (i) experimentally test whether different amounts and forms of P fertilizer affect AMF diversity and composition in the roots of apple trees; and (ii) identify differences in tolerance to P fertilization among different AMF taxa. To this end, we used high-throughput pyrosequencing data from a total of 24 apple root samples obtained from a three year long field experiment with two inorganic and three slow-release P fertilization treatments.

4.3 Materials and Methods

4.3.1 Field experiment

Our study site was an experimental apple orchard at the PCFruit research station located near Sint-Truiden, Belgium (50° 46' 22" N, 5° 9' 36" E). This orchard is characterized by a loamy soil and a maritime temperate climate with an average yearly rainfall of 800 mm and an annual average temperature of 9.8 °C (Royal Meteorological Institute, Ukkel, Belgium). All apple trees were planted in 2009. The trees were of the Jonagold cultivar and grafted on M9 rootstocks, the most commonly used rootstock for commercial apple production in Belgium. The interrow distance was 3.25 m and the distance between trees in the row was 1.25 m.

The experiment, that started in August 2011, included six fertilization treatments and two fertilizer types, including two quick-release inorganic fertilizers and three slow-release fertilizers. The following six treatments were included in the study: (1) Control (CO), addition of 0 kg P_2O_5 / ha; (2) Inorganic fertilizer 20 (IF20), addition of 20 kg P_2O_5 / ha; (3) Inorganic fertilizer 50 (IF50), addition of 50 kg P_2O_5 / ha; (4) Mushroom compost (MC), addition of 28 kg / ha P_2O_5 mushroom compost; (5) Green compost (GC), addition of 30 kg / ha P_2O_5 green compost; (6) Struvite (ST), addition of 20 kg / ha P_2O_5 struvite. As the organic treatments have a fixed N:P:K ratio, the small differences in P_2O_5 levels among the slow-release treatments were meant to avoid an excess in N and K addition, which would damage fruit production of the trees. Each treatment was replicated four times and each replicate consisted of five neighboring apple trees. The different replicates were randomly distributed throughout the orchard to avoid positioning effects. Fertilizers were applied annually in March or May. The quick-release fertilizers (treatment 2 and 3) consisted of freely available phosphate (P_2O_5). Mushroom compost is the residual compost waste generated by the mushroom production industry and generally consists of a combination of wheat straw, horse manure and ground chalk. Green compost is the end product of the composting of organic waste (pruning wood, leaves and grass cuttings) from gardens, parks and public green spaces. Struvite (magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6(H_2O)$) contains 5.7 % N, 12.6 % P and 9.9 % Mg. When applied as a fertilizer, it will slowly dissolve and release its components. Calcium nitrate was added to the inorganic treatments in order to equalize the amount of nitrogen at 30 kg / ha NO_3^- for all treatments. To compensate for the extra potassium in the treatments with organic fertilizer, we also equalized the potassium amount for the inorganic treatments at 30 kg K / ha.

In August 2014, fine root and soil samples were taken from each of the 5 apple trees per replicate (Guo *et al.*, 2008). Samples were pooled for each replicate, resulting in four root samples per treatment. In parallel, one soil sample per replicate, consisting of 25 pooled soil cores (10 cm depth, 3.5 cm diameter), was taken around the five apple trees for chemical analysis, also resulting in four soil samples per treatment. In total, 24 root samples and 24 soil samples were obtained. Root and soil samples were stored at 4 °C until further use.

4.3.2 Soil chemical analysis

Soil pH was determined using a pH probe in a 1:10 soil/water mixture. As a measure of the plant-available N content of the soil, ammonium and nitrate availability was determined by shaking 10 g of soil in 200 mL of 1 M potassium chloride solution for one hour. Extracts were analyzed colorimetrically using a segmented flow auto analyzer (Skalar, Breda, the Netherlands). As a measure of the plant-available P content of the soil, Olsen P values were determined by shaking 2 g dry soil for 30 minutes with 0.5 M sodium bicarbonate at pH 8.5 and subsequent colorimetric analysis of the extracts using the molybdenum blue method (Robertson *et al.*, 1999). Organic carbon content was determined by shaking 10 g of soil in an excess volume of 0.27 M potassium dichromate and 18 M sulfuric acid at a temperature of 135 °C. Extracts were analyzed colorimetrically.

4.3.3 DNA extraction, PCR amplification and pyrosequencing

The obtained root samples were cut in 1 cm pieces and rinsed twice with sterile distilled water. For each sample, 0.1 g root material was used to extract DNA using the UltraClean Plant DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer's instructions. Subsequently, the obtained DNA was diluted 10 times prior to PCR amplification. PCR amplification was performed using primer pair AMV4.5NF-AMDGR (Sato *et al.*, 2005), as this primer pair is highly AMF specific and is able to consistently describe AMF communities using 454 pyrosequencing based on the most variable part of the small subunit (SSU) rRNA gene region (Van Geel *et al.*, 2014). 'Fusion' primers, required for the 454 pyrosequencing process, were designed according to the guidelines for 454 GS-FLX Titanium Lib-L sequencing and contained the Roche 454 pyrosequencing adapters and a sample-specific MID barcode in between the adapter and the forward primer. In total, 24 MID barcodes (recommended by Roche, Mannheim, Germany) were used for sample-specific amplicon tracking. PCR reactions were performed on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) in a reaction volume of 20 µl, containing 0.15 mM of each dNTP, 0.5 µM of each primer, 1x Titanium *Taq* PCR buffer, 1U Titanium *Taq* DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), and 1 µl genomic DNA. Before amplification, DNA samples were denatured at 94°C for 2 min. Next, 30 cycles were run, consisting of 45 s at 94°C, 45 s at 65°C and 45 s at 72°C, followed by a final elongation of 10 min at 72°C. After resolving the amplicons by agarose gel electrophoresis, amplicons within the appropriate size range were cut from the gel and purified using the Qiaquick gel extraction kit (Qiagen, Hamburg, Germany). Purified dsDNA amplicons were quantified using the Quant-iT PicoGreen® dsDNA Assay Kit and the Qubit fluorometer (both from Invitrogen, Ghent, Belgium), and pooled in equimolar quantities. The quality of the amplicon library was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The amplicon library was loaded on $1/4^{th}$ of a 454 Pico Titer Plate and pyrosequencing was performed using the Roche GS-FLX instrument and Titanium chemistry according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

4.3.4 Bioinformatics

Sequences obtained from the 454 pyrosequencing run were clustered into operational taxonomic units (OTUs) using the UPARSE algorithm, following the recommended pipeline (Edgar, 2013). First, quality filtering of the reads was performed with the 'fastq_filter' command, allowing a maximum expected error of 0.5 for the individual sequences. In order to maximize the number and length of retained sequences, truncation length was set to 233 bp. Next, the sequences were dereplicated and sorted by abundance. Subsequently, singletons, i.e. sequences only occurring once in the entire dataset, were removed prior to clustering as this has been shown to improve the accuracy of diversity estimates (Kunin *et al.*, 2010; Tedersoo *et al.*, 2010a; Waud *et al.*, 2014). Then, sequences were clustered into OTUs defined at 97% sequence similarity, which is commonly used to define SSU-based OTUs in AMF (Lumini *et al.*, 2010; Öpik *et al.*, 2010), with the 'cluster_otus' command. In this step, chimeric OTUs built from more abundant reads were discarded as well. However, few chimeras may be missed if their parents were absent or present with very low abundance.

Therefore, obtained OTUs were double-checked for chimeric sequences against the MaarjAM database (Öpik *et al.*, 2010) using the 'uchime_ref' command. OTUs were assigned to a taxonomic identity by querying the representative sequence (as determined by the 'cluster_otus' command) against GenBank using the BLAST algorithm (Altschul *et al.*, 1990). Taxonomic assignments were considered reliable when a ≥200 BLAST score value was found (Lumini *et al.*, 2010). OTUs not belonging to the Glomeromycota or having a BLAST score lower than 200 were discarded. To accurately identify the obtained AMF OTUs, the representative sequence for each OTU was also queried against the MaarjAM database (Öpik *et al.*, 2010; accessed 3 February 2015), a database that aims to provide a quality-controlled repository for published sequence data from Glomeromycota.

4.3.5 Data analysis and statistics

To assess the sampling effort, rarefaction curves were calculated in MOTHUR (Schloss *et al.*, 2009) for each treatment, using re-sampling without replacement. To prevent bias due to different sequencing depth, all samples were subsequently rarefied to the lowest sequencing depth per sample. Samples with less than 1000 sequences were removed.

AMF richness was determined as the number of AMF OTUs present in a sample. AMF diversity was approximated by the Shannon index (H) and was calculated using the 'summary.shared' command in MOTHUR. The Shannon index was exponentially transformed to obtain a true diversity estimate (Exp(H)). After this conversion, AMF diversity is measured in units of number of OTUs and the variable behaves linear, in contrast to the non-transformed Shannon diversity (see Jost (2006) for details). First, we used mixed models to test for differences in AMF richness and Exp(H) between the two main fertilizer types (inorganic versus slow-release treatments). Here, we included the separate fertilization treatments as a random factor to account for the effect of treatment within a fertilizer type. Subsequently, differences in AMF richness and Exp(H) were tested between all treatments, using a one-way ANOVA. These analyses were performed in SPSS 20.0 (SPSS Inc., Chicago, IL).

To test the effect of fertilizer types and of all treatments on AMF community composition, we performed a non-metrical multidimensional scaling (NMDS) on the sample*OTU matrix, using Bray-Curtis distances (R- package Vegan, Oksanen *et al.*, 2013). Subsequently, differences in community composition between all treatments and fertilizer types were tested for significance based on a permutation test with 1000 iterations, using the function 'envfit' (Vegan package). Ellipses were plotted on the ordination representing communities belonging to the different treatments using the 'ordiellipse' function (Vegan package) using the standard deviation of the mean.

To detect OTUs specific for any of the fertilizer types or treatments, we performed an indicator species analysis in PC-ORD 6 (Dufrêne & Legendre, 1997; McCune & Mefford, 2006). This analysis calculates an indicator value based on fidelity and relative abundance of an OTU in relation to the treatment classes. By definition, an indicator value of 100 (perfect indicator OTU) implies that the presence of a given OTU identifies a treatment without error. The obtained indicator values were tested for significance using a Monte Carlo randomization test with 1000 permutations.

Finally, to test whether AMF communities showed a nested structure, i.e. to test whether AMF OTUs detected in OTU-poor samples are a subset of the OTUs found in OTU-rich samples, a formal nestedness analysis was performed using BINMATNEST (Rodriguez-Girones & Santamaria, 2006), as implemented in the nestedness function in the bipartite package of R (Dormann *et al.*, 2008). This function calculates the temperature of the presence/absence sample*OTU matrix, a measure of nestedness varying between 0° (perfectly nested) and 100° (perfectly non-nested) and estimates whether the input matrix is more nested than expected by chance. The significance of nestedness was tested using default input parameters and null model 3. In order to assess whether the communities of certain treatments or fertilizer types were nested within others, the positions of the samples representing the different treatments in the maximally stacked matrix were compared through a non parametric Kruskal-Wallis test.

4.4 Results

4.4.1 Soil analysis

As expected, pH, nitrate and ammonium concentrations in the soil were found to be similar for the six fertilization treatments (Table 4.1). In contrast, the soil phosphate and organic matter content differed considerably. The IF50 treatment had on average the highest phosphate levels, followed by the IF20 treatment. The CO and slow-release fertilizer treatments had similar phosphate levels ranging from 38.7 to 49.8 mg P / kg. The treatments where organic fertilizer was applied (MC and GC treatments) showed on average a higher organic matter content in comparison to the other treatments, except for the IF20 treatment. Furthermore, the organic matter content was similar for the CO, IF50 and ST treatment (Table 4.1).

Table 4.1 Soil chemical properties of the six fertilization treatments (N = 4; SE = standard error). Concentrations of ammonium, nitrate and phosphate are presented as atomic weight fractions (of N or P in mg/kg). Organic carbon (OC) content is expressed as a percentage.

	pH		NH_4^+		NO_3^-		PO_4^{2-}		OC	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	6.5	0.1	6.5	4.2	16.9	0.6	40.8	2.3	4.0	0.3
Inorganic fertilizer 20	6.5	0.0	1.6	0.2	19.0	1.9	59.0	11.5	4.8	0.1
Inorganic fertilizer 50	6.6	0.1	16.3	14.4	15.1	1.9	79.0	7.5	4.2	0.3
Mushroom compost	6.6	0.1	5.9	2.9	22.2	2.8	48.3	7.6	4.9	0.2
Green compost	6.5	0.1	2.2	0.5	21.3	0.7	49.8	2.8	5.1	0.3
Struvite	6.5	0.1	2.6	0.4	16.7	1.7	38.7	4.7	4.0	0.2

4.4.2 Pyrosequencing

Pyrosequencing resulted in a total of 64 453 reads with a minimal length of 233 bp and containing the correct barcode and primer sequence, ranging from 225 to 8 213 reads per sample. A BLAST search against GenBank revealed the presence of 61 169 (94.9 %) Glomeromycota reads, ranging from 206 to 7 630 AMF reads per sample. Subsequently, we rarefied to 1399 AMF reads per sample, leaving 22 of the 24 initial samples and 30 778 reads for further analysis.

4.4.3 AMF diversity

In total, 39 AMF OTUs were detected in the rarefied dataset. The majority of OTUs belonged to the Glomeraceae (72 %, 28 OTUs, 19 543 sequences) and Claroideoglomeraceae (26 %, 10 OTUs, 11 229 sequences), whereas only one OTU belonged to the Paraglomeraceae (2 %, 1 OTU, 6 sequences) (Appendix Table C1). All rarefaction curves tended to saturate at the chosen sequencing depth (4 times 1399 sequences per sample). This was also the case for the IF20 and the MC treatment that consisted of only three samples after rarefying (Appendix Fig. C1). In total, more OTUs were observed in the slow-release fertilizer treatments (27, 30 and 32 OTUs) compared to the inorganic fertilizer treatments (17 and 24 OTUs); in the CO treatment 25 OTUs were found (Appendix Fig. C1). Significant differences in AMF richness were observed between inorganic and slow-release fertilization treatments ($F_{2, 19}$ = 5.615, P = 0.012). The slow-release fertilizer treatments had a significantly higher AMF richness than the inorganic fertilizers and the control (Table 4.2). Although no significant differences were found in Shannon diversity between treatments ($F_{5, 16}$ = 1.971, P = 0.138) or fertilizer types ($F_{2, 19}$ = 3.277, P = 0.060), our results showed a trend towards higher Shannon diversity values in the slow-release fertilizer treatments (Table 4.2; $F_{5, 16}$ = 3.277, P = 0.060). The analysis of six separate treatments showed AMF richness to be marginally significantly different between different fertilizer treatments ($F_{2, 19}$ = 2.775, P = 0.054). Tukey's HSD test revealed that AMF richness significantly increased in the ST treatment compared to the IF20 treatment (P = 0.049) (Table 4.2).

Table 4.2 AMF richness and Shannon diversity (H) in the roots of apple trees after 3 years of different fertilization treatments (SE = standard error). Different letters indicate significant differences among fertilizer types and treatments, as determined by Tukey's HSD test.

			AMF richness		Exp(H)	
		N	Mean	SE	Mean	SE
Fertilizer Type	Control	4	14.75^{a}	0.48	4.47	0.46
	Inorganic fertilizer	7	13.86^{a}	0.94	4.29	0.46
	Slow-release fertilizer	11	18.36^{b}	1.07	5.72	0.41
Treatment	Control	4	14.75^{ab}	0.48	4.47	0.46
	Inorganic fertilizer 20	3	12.67^{a}	0.67	4.86	0.53
	Inorganic fertilizer 50	4	14.75$_{ab}$	1.49	3.86	0.66
	Mushroom compost	3	17.67$_{ab}$	0.88	5.74	0.84
	Green compost	4	17.25^{ab}	2.39	6.35	0.78
	Struvite	4	20.00^{b}	1.73	5.07	0.56

4.4.4 AMF community composition

The NMDS permutation test showed that AMF communities differed between the two different fertilizer types (R^2 = 0.27, P = 0.01) (Fig. 4.1). AMF community composition from the slow-release treatments were clearly distinct from the inorganic fertilization treatments (Fig. 4.1). Samples from the CO treatment, took an intermediate position in the NMDS ordination (Fig. 4.1). However, no significant differences in AMF community composition between the six fertilization treatments were found (R^2 = 0.34, P = 0.11).

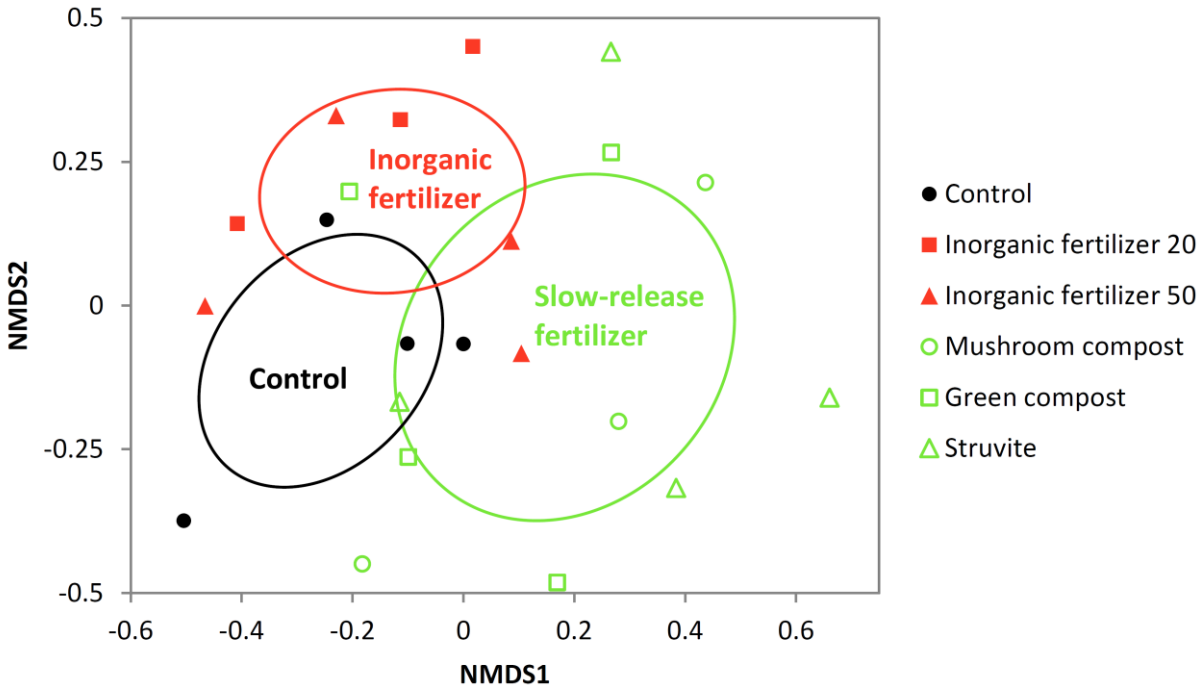


Figure 4.1 Non-metric multidimensional scaling ordination plot of arbuscular mycorrhizal fungal communities in the roots of apple trees treated with different fertilizer types. Arbuscular mycorrhizal fungal communities treated with inorganic fertilizers (closed squares and triangles) were significantly different from those treated with slow-release fertilizers (open cirkels, squares and triangles) (R^2 = 0.27, P = 0.01). Ellipses are dispersion ellipses using the standard deviation of the mean. All samples were rarefied to 1399 AMF reads per sample, leaving 22 of the 24 initial samples. Stress value: 18.1.

4.4.5 Indicator species analysis

The indicator species analysis detected one indicator OTU significantly associated with the slow-release treatments (OTU_06). Similarly, one OTU was significantly indicative of the CO treatment (OTU_27) and one OTU was a significant indicator of the ST treatment (OTU_03) (Table 4.3). Indicator OTU_27 and OTU_06 belonged to the family Glomeraceae (genus *Glomus*), while the indicator OTU_03 belonged to the Claroideoglomeraceae (genus *Claroideoglomus*).

Table 4.3 Indicator OTUs detected for the different fertilizer types and treatments. Significance levels are obtained by Monte Carlo permutation tests.

	OTU ID	Group	Number of reads[a]	Indicator value[b]	*P*
Fertilizer Type	OTU_06	Slow-release fertilizer	811	70.2	0.045
Treatment	OTU_27	Control	129	98.1	0.004
	OTU_03	Struvite	2772	51.1	0.023

[a] Rarefied dataset
[b] OTUs with non-significant indicator values are not shown

4.4.6 Nestedness

The distribution of AMF OTUs showed a nested pattern, as indicated by a matrix temperature of 29.1, which was significantly lower than expected by chance (expected T = 48.3, P < 0.001). The position of the samples from the two fertilizer types differed significantly from each other in the stacked minimum temperature matrix (Kruskal-Wallis H = 10.3 and *P* = 0.006), while the positions of the six different fertilization treatments did not (Fig. 4.2). More specifically, samples from the inorganic fertilizer treatments were positioned significantly higher than those of the slow-release treatments (Kruskal-Wallis $Z_{22,2} = 2.97$, *P* = 0.009). Therefore, samples from the inorganic fertilizer treatments can be considered to be a subset of the slow-release treatments.

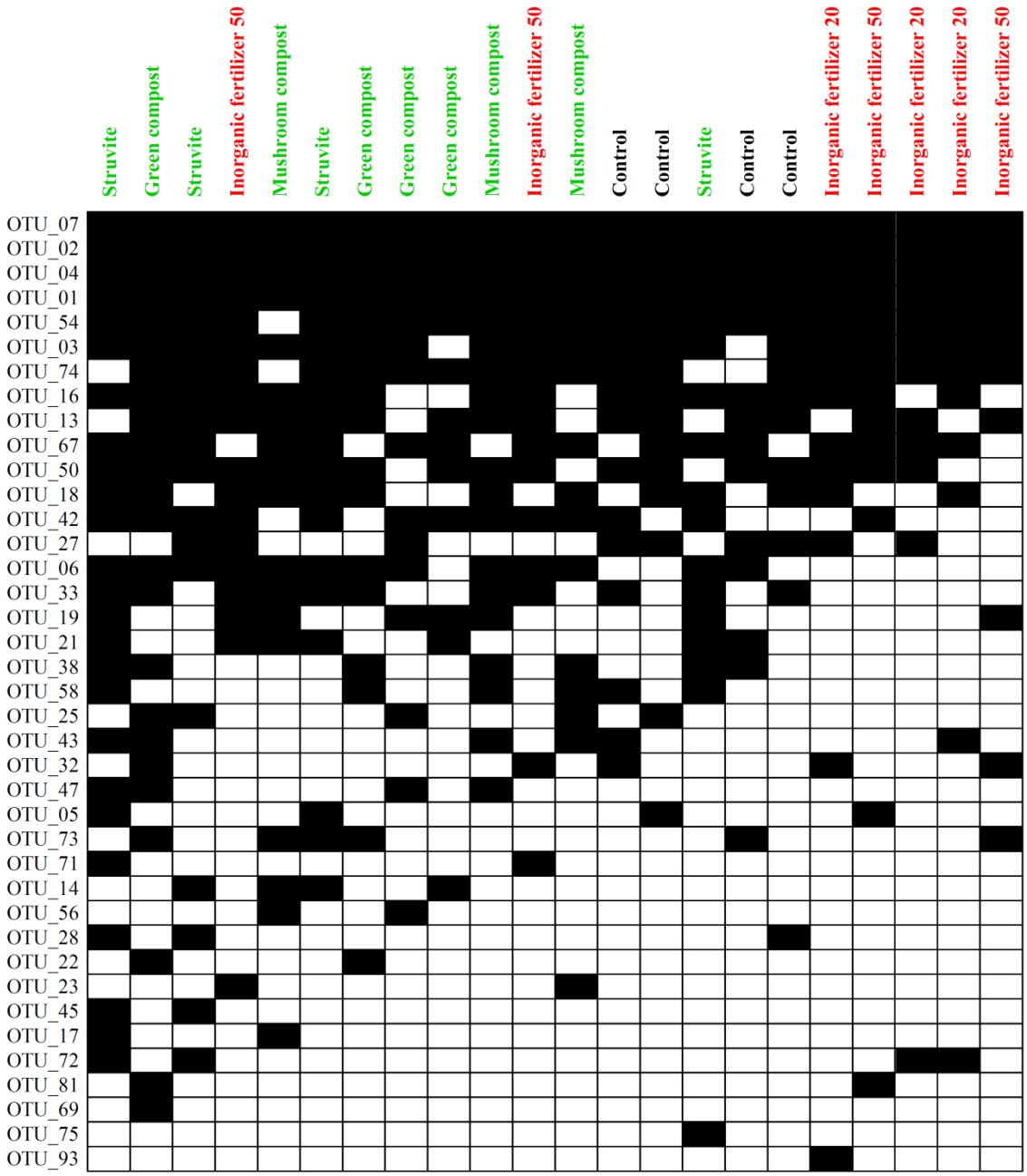


Figure 4.2 Nestedness of arbuscular mycorrhizal fungal communities in the roots of apple trees treated with different types of fertilization as shown by the minimum temperature matrix. Arbuscular mycorrhizal fungal communities treated with inorganic fertilizers (red) were significantly more nested compared with those treated with slow-release fertilizers (green) (Kruskal-Wallis Z = 2.97, *P* = 0.009). Columns represent samples (sorted according to their degree of nestedness) and rows are OTUs.

4.5 Discussion

Our results showed a significantly higher AMF richness in the slow-release fertilizer treatments compared to the inorganic fertilizer treatments. We found a marginally significant trend towards higher Shannon diversity values in the slow-release fertilization treatments. Given the lower plant-

available P in the slow-release fertilization treatments, these results suggest that slow-release fertilizers promote AMF diversity through controlling the plant-available P levels in the soil. Indeed, a significantly negative correlation was observed between the plant-available P in the soil and the AMF richness (r = -0.531, P = 0.011), whereas other soil variables did not correlate with AMF richness.

The NMDS analysis showed that AMF communities differed significantly between the inorganic and slow-release fertilizer types, suggesting that different P fertilization applications can induce a shift in AMF community composition. This supports former findings that the abundance of soil nutrients plays a crucial role in shaping the intraradical AMF community. These results can be explained by the elevated plant-available P levels in the soil in the inorganic P fertilization treatments. Indeed, it has been shown that plants will allocate biomass to the structures that best garner the most limiting resources (Johnson *et al.*, 2013). Therefore, elevated plant-available P levels through inorganic P fertilization will reduce plant allocation to roots and negatively affect the mycorrhizal symbiosis through increasing the competition for carbohydrates between AMF taxa. Consequently, those AMF taxa that are the most efficient in acquiring plant carbohydrates under elevated plant-available P levels in the soil will be selected (Werner & Kiers, 2015b), resulting in a shift in community composition. In agreement with this, we also found that the AMF communities detected in the different fertilization treatments were organized in a nested pattern. More specifically, we observed that the poorer AMF communities in the inorganic fertilizer treatments were nested within the richer AMF communities in the slow-release fertilizer treatments. As already suggested, increased competition through elevated phosphate availability will favor those AMF taxa that are the most efficient in acquiring plant carbohydrates. Consequently, the more sensitive AMF taxa may disappear in the inorganic fertilization treatments and cause community nestedness.

By using indicator species analysis, we found that OTU_06 (which was identified as virtual taxa VTX00214 in the MaarjAM database, Öpik *et al.*, 2010) was an indicator of the slow-release fertilization treatments. This virtual taxon has not only been reported from several anthropogenic systems (Balestrini *et al.*, 2010), but also from natural ecosystems, such as grasslands and forests (Öpik *et al.*, 2003). It was also found in the roots of other crops such as *Zea mays*, *Triticum aestivum* and *Glycine max*, although always in soils with low available P levels (11-12 mg/kg) (e.g. Liu *et al.*, 2012; Beauregard *et al.*, 2013). These findings suggest that VTX00214 is sensitive to higher plant-available P levels in the soil. Additionally, we found that OTU_27 (which was identified as virtual taxa VTX00115 in the MaarjAM database) was an indicator of the CO treatment. This virtual taxon has also been observed in grasslands (Öpik *et al.*, 2003) and forests (Öpik *et al.*, 2009), but also in anthropogenic systems, such as a peach orchard (Alguacil *et al.*, 2011). Therefore, this taxon may be well adapted to agricultural conditions.

No OTUs were found that were indicative for the inorganic fertilization treatments. This may be explained by the nested pattern of our dataset which indicates that taxa from the local taxa pool are filtered out under specific conditions. Therefore, no specific OTUs, but rather generalist AMF taxa, are expected in the inorganic fertilization treatments. Indeed, four OTUs were observed in all samples and treatments, including the inorganic fertilization treatments (OTU_01 (VTX00057),

OTU_02 (VTX00113), OTU_04 (VTX00163) and OTU_07 (VTX00163)). Of these four OTUs, OTU_02 (VTX00113) was the most abundant. In the MaarjAM database, VTX00113 is also the most abundant taxon and in some entries it has been identified as *Rhizophagus intraradices*. This is one of the most widespread AMF taxa and has been detected in different continents and ecosystems, from arable fields to grasslands and forests. It has also been observed in high-input agricultural fields (Hijri *et al.*, 2006), suggesting it is tolerant to high nutrient levels in the soil. Additionally, sporulation of *R. intraradices* has been shown to be insensitive to P fertilization (Sylvia & Schenk, 1983). Moreover, Johnson (1993) reported that after eight years of fertilizer application, the proliferation of generalistic AMF mutualists, such as *R. intraradices*, in the roots of big bluestem grass may be favored. It has been shown that *R. intraradices* is an inferior mutualist that can negatively affect the growth of citrus trees grown in a soil with high plant-available P (Peng *et al.*, 1993). Our results thus suggest that the application of inorganic fertilizers may lead towards communities dominated by inferior AMF mutualists.

Overall, our results emphasize that the application of slow-release fertilizers can promote AMF diversity in the roots of the host. Inorganic fertilizers on the contrary, may lower the AMF diversity and result in AMF communities dominated by inferior AMF mutualists due to elevated levels of plant-available P in the soil. Our results thus suggest important additional benefits of using slow-release fertilizers, such as green compost, mushroom compost and struvite.

Chapter 5

Available phosphorus rather than management or copper drives arbuscular mycorrhizal communities in Flemish vineyards

5.1 Summary

AMF are one of the most important components in agricultural ecosystems and form a symbiosis with the majority of the land plants, including grapevine. For normal growth and development, grapevines depend on AMF that increase nutrient uptake and tolerance to abiotic and biotic stress. The objective of this study was to determine which factors (soil chemical variables, management and geography) relate to AMF diversity and community composition, determine how elevated copper concentrations in the soil affect AMF communities, and identify differences in tolerance to nutrient levels in the soil between AMF taxa. To accomplish this, we used high-throughput pyrosequencing on 170 root samples from grapevines originating from 18 conventionally and 16 organically managed vineyards. We found no differences in AMF diversity between organically and conventionally managed vineyards. Instead, plant-available P content of the soil and pH were the only variables significantly related to AMF diversity. In agreement with our diversity analysis, the unconstrained and constrained ordination approach revealed that the available P content in the soil was significantly related to AMF community composition. The effect of management type on AMF community composition, however, was unclear, indicating management type could explain very little variation in AMF community composition. Although we found no effects of copper concentration in the soil on AMF diversity or community composition, we observed that older vineyards (> 15 years) showed copper concentrations above the background level (30 mg/kg). We conclude that any positive effects of organic management on AMF diversity in vineyards were overruled by soil characteristics. Organic management is thus no guarantee for high AMF diversity, as the soil of organically managed vineyards may still contain high available P levels. In addition, higher plant-available P levels and lower pH levels may lead to AMF communities dominated by generalists and a gradual loss of specialist AMF taxa sensitive to high nutrients in the soil and soil acidity.

5.2 Introduction

Grapevine (*Vitis vinifera*) is a perennial crop, grown worldwide on an area of 7.53 million ha. In 2015, world wine production reached 274.4 Mhl and wine traded for a total of 28.3 billion Euro (OIV, 2015). Grapevine is highly responsive to management practices and local environmental conditions and therefore even different vineyards of the same cultivar may produce a distinctive grape characteristics. This feature of grapevines is comprised in the term 'terroir', which has been defined as "a concept which refers to an area in which collective knowledge of the interactions between the identifiable physical and biological environment and applied vitivinicultural practices develops, providing distinctive characteristics for the products originating from this area" (Resolution OIV/viti 333/2010). Many studies have investigated the effect of physical environmental conditions and management practices on grape composition and wine quality (reviewed in Jackson & Lombard, 1993). Recently, Gilbert *et al.* (2014) suggested that the microbial communities that coexist with grapevines may be among the key factors that influence grape characteristics, referring to a 'microbial terroir'. Also soil microbial associated with grapevine may be important in this context, although these remain less intensively studied (Trouvelot *et al.*, 2015).

Among soil microorganisms, arbuscular mycorrhizal fungi (AMF) are key components in agricultural ecosystems and form a symbiosis with the majority of the land plants, including grapevine (Smith & Read, 2008). In return for plant photosynthates, AMF provide a range of benefits to the grapevine through their extraradical hyphal network, which acts as a living interface between the roots and the soil. Numerous studies have shown that grapevines depend on AMF for normal growth and development (Schreiner, 2005). AMF mainly increase phosphorus (P) and nitrogen (N) uptake by grapevines, but increased uptake of other nutrients, such as zinc, copper, potassium and calcium, also have been found (Schreiner, 2005). AMF can also enhance grapevine tolerance to abiotic stress conditions, such as drought (Valentine *et al.*, 2006), salinity (Belew *et al.*, 2010; Khalil, 2013) or heavy metals (Karagiannidis & Nikolaou, 2000). Furthermore, AMF can protect grapevine from soil-borne pathogens (Petit & Gubler, 2006; Hao *et al.*, 2012) and stabilize the soil through entangling soil particles with their hyphae (Rillig & Mummey, 2006). Given both the potential importance of microbial terroir and the beneficial effects of AMF on grapevine, it is crucial to understand how environmental conditions and management practices can influence AMF communities in the roots of grapevine.

High fertilizer inputs have widely been recognized to negatively affect AMF abundance (Kahiluoto *et al.*, 2001; Jansa *et al.*, 2009). In grapevine, it has been shown that high soil P levels reduce AMF root colonization (Karagiannidis & Nikolaou, 1999). Also grapevine N fertilization suppressed AMF colonization and sporulation (Karagiannidis *et al.*, 2007). How AMF communities in the roots of grapevine changes with increasing soil P or N levels, and which AMF taxa are most susceptible to fertilization is, however, still poorly understood.

Since the end of the nineteenth century, copper sulfate (Bordeaux mixture) has been used in vineyard soils to control vine fungal diseases, such as Downy mildew (*Plasmopara viticola*), and led

to a widespread accumulation of copper. Whereas normal background concentrations of copper range from 5-30 mg kg^{-1}, copper concentrations ranging from 100 up to 1500 mg kg^{-1} have been measured in European vineyards with a long history of copper-based fungicide use (Deluisa *et al.*, 1996; Flores-Vélez *et al.*, 1996). High copper concentrations in the soil have been shown to negatively affect a wide range of soil biota in agricultural ecosystems (Korthals *et al.*, 1996; Van Zwieten *et al.*, 2004; Bunemann *et al.*, 2006). To our knowledge, however, no such evidence exists for AMF communities.

Apart from soil nutrient levels and copper concentrations, also the type of agricultural management can affect AMF communities. Compared to conventional agricultural management, organic management, which typically excludes the use of chemical fertilizers and pesticides, has already been shown to increase the diversity of a wide range of taxa, including birds, mammals, invertebrates and plants (Hole *et al.*, 2005; Tuck *et al.*, 2014). Although organic farming has also been shown to be beneficial for AMF diversity in a wide range of crops such as potato and apple (Verbruggen *et al.*, 2010; Van Geel *et al.*, 2015), such evidence for vineyards is still scarce. The few studies that have investigated AMF communities in vineyards were performed on a relatively small scale or used fingerprinting methods, which lack sufficient resolution to thoroughly characterize AMF diversity. For example, Lumini *et al.* (2010) studied the AMF communities in vineyards and agricultural fields, and showed that AMF communities mirrored the land-use gradient. Only two vineyards, however, were sampled in this study. Moreover, Balestrini *et al.* (2010) identified AMF originating from only two Italian vineyard soils using morphological characteristics together with sanger sequencing. Furthermore, Likar *et al.* (2013) investigated AMF communities in the roots of grapevines along the eastern Adriatic coast based on eight vineyard sites, but used Temperature Gradient Gel Electrophoresis (TTGE) analysis. Studies identifying the environmental drivers of AMF communities in vineyards using high-throughput sequencing methods at a large scale are thus still lacking.

The objective of this study was to (i) characterize AMF communities in the roots of grapevines across 34 vineyards located in the Flanders region (N-Belgium), and the most Southern part of the Netherlands; (ii) determine which factors (soil chemical variables and management type (conventional *vs.* organic)) relate to AMF diversity and community composition; (iii) determine how elevated copper concentrations in the soil affect AMF communities; (iv) detect specific AMF taxa for organic and conventional vineyards; and (v) identify differences in tolerance to high fertilization levels between AMF taxa. To accomplish this, we used high-throughput pyrosequencing on 170 root samples from grapevines originating from 18 conventionally and 16 organically managed vineyards.

5.3 Materials and methods

5.3.1 Study sites and sampling

The study was conducted in Flanders, the northern part of Belgium. This region has a maritime mesothermic climate with significant precipitation in all seasons. Annual average precipitation is 785 mm and average annual temperature is 9.8°C (Royal Meteorological Institute, Ukkel, Belgium). A total of 34 vineyards were examined within this study (average distance between vineyards was 87.9 km, minimal 1 km, maximal 223 km) (Appendix Fig. D1 and Table D1). Three vineyards were located in the Netherlands, just across the Flemish border (Vineyard 30, 31 and 32) (Appendix Fig. D1). A stratified random sampling design, stratified by the type of management, was used. We sampled 18 conventionally and 16 organically managed vineyards across Flanders (Appendix Fig. D1). In the organic vineyards, no chemical fertilizers, or pesticides were used since transformation to organic management. However, organic fertilizers and small amounts of copper-based fungicides to control Downy mildew (*Plasmopara viticola*) were allowed. The relative size (highest value divided through the lowest value) of the sampled soil gradient was 1.43 for pH (logarithmic scale), 22.08 for Soil N, 34.12 for organic carbon content, and 41.53 for Olsen P. Planting density or plant age did not differ between both types of vineyards. All grapevines were grafted on SO4 rootstocks, a frequently used rootstock for commercial grapevine production. In October 2015, roots from five randomly chosen grapevines per vineyard were excavated. Root samples were collected on three random locations of the root system and were pooled afterwards to obtain one pooled root sample per grapevine. Especially fine roots were collected, as these are known to contain AMF (Guo *et al.*, 2008). A soil sample for chemical analysis was also collected near each sampled individual. Root and soil samples were stored at 4 °C until further analysis. In total, 170 root and soil samples across 34 vineyards were obtained.

5.3.2 Soil chemical analysis

Soil pH was quantified using a pH probe in a 1:10 soil/water mixture. As a measure of the plant-available N content of the soil, ammonium and nitrate availability was quantified by shaking 10 g of soil in 200 mL of 1 M potassium chloride solution for one hour. Extracts were analyzed colorimetrically using a segmented flow auto analyzer (Skalar, Breda, the Netherlands). As a measure of the plant-available P content of the soil, Olsen P values were quantified by shaking 2 g dry soil for 30 minutes with 0.5 M sodium bicarbonate at pH 8.5 and subsequent colorimetric analysis of the extracts using the molybdenum blue method (Robertson *et al.*, 1999). Organic carbon content was quantified by shaking 10 g of soil in an excess volume of 0.27 M potassium dichromate and 18 M sulfuric acid at a temperature of 135 °C. Extracts were analyzed colorimetrically. Copper concentration in the soil was measured by digesting 50 mg of dried and sieved soil with 7.5 ml concentrated hydrochloric acid and 2.5 ml concentrated nitric acid. The digested solution was diluted to 10 ml and measured with ICP-OES.

5.3.3 DNA extraction, PCR amplification and pyrosequencing

Roots samples were cut in 1 cm pieces and rinsed twice with sterile distilled water. For each sample, 0.1 g root material was used to extract DNA, using the UltraClean Plant DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer's instructions. Subsequently, the obtained DNA was diluted 10 times prior to PCR amplification. PCR amplification was performed using primer pair AMV4.5NF-AMDGR (Sato *et al.*, 2005), as this primer pair is highly AMF specific and is able to consistently describe AMF communities using 454 pyrosequencing based on the most variable part of the small subunit (SSU) rRNA gene region (Van Geel *et al.*, 2014). 'Fusion' primers, required for the 454 process, were designed according to the guidelines for 454 GS-FLX Titanium Lib-L sequencing containing the Roche 454 pyrosequencing adapters and a sample-specific MID barcode in between the adapter and the forward primer. In total, 57 MID barcodes (recommended by Roche, Mannheim, Germany) were used for sample-specific amplicon tracking. PCR reactions were performed on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) in a reaction volume of 20 µl, containing 0.15 mM of each dNTP, 0.5 µM of each primer, 1x Titanium *Taq* PCR buffer, 1U Titanium *Taq* DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), and 1 µl genomic DNA. Before amplification, DNA samples were denatured at 94°C for 2 min. Next, 35 cycles were run, consisting of 45 s at 94°C, 45 s at 65°C and 45 s at 72°C, followed by a final elongation of 10 min at 72°C. After resolving the amplicons by agarose gel electrophoresis, amplicons within the appropriate size range were cut from the gel and purified using the Qiaquick gel extraction kit (Qiagen, Hamburg, Germany). Purified dsDNA amplicons were quantified using the Quant-iT PicoGreen® dsDNA Assay Kit and the Qubit fluorometer (both from Invitrogen, Ghent, Belgium), and pooled in equimolar quantities over three amplicon libraries, each representing 57 samples tagged with a unique MID barcode. The quality of the amplicon libraries was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The amplicon libraries were each loaded on a $1/4^{th}$ of a 454 Pico Titer Plate and pyrosequencing was performed using the Roche GS-FLX instrument and Titanium chemistry according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

5.3.4 Bioinformatics

Sequences obtained from the 454 pyrosequencing run were clustered into operational taxonomic units (OTUs) using the UPARSE algorithm, following the recommended pipeline (Edgar, 2013). First, quality filtering of the reads was performed with the 'fastq_filter' command, allowing a maximum expected error of 0.5 for the individual sequences. In order to maximize the number and length of retained sequences, truncation length was set to 225 bp. Next, the sequences were dereplicated and sorted by abundance. Subsequently, singletons, i.e. sequences only occurring once in the entire dataset, were removed prior to clustering as this has been shown to improve the accuracy of diversity estimates (Tedersoo *et al.*, 2010a; Waud *et al.*, 2014; Brown *et al.*, 2015). Then, sequences were clustered into OTUs defined at 97% sequence similarity, which is commonly used to define SSU-based OTUs in AMF (Lumini *et al.*, 2010; Öpik *et al.*, 2010), with the 'cluster_otus' command. In this step, chimeric OTUs built from more abundant reads were discarded as well. However, few

chimeras may be missed if their parents were absent or present with very low abundance. Therefore, obtained OTUs were double-checked for chimeric sequences against the MaarjAM database (Öpik *et al.*, 2010) using the 'uchime_ref' command. OTUs were assigned to a taxonomic identity by querying the representative sequence (as determined by the 'cluster_otus' command) against GenBank using the BLAST algorithm (Altschul *et al.*, 1990). Taxonomic assignments were considered reliable when a ≥200 BLAST score value was found (Lumini *et al.*, 2010). OTUs not belonging to the Glomeromycota or having a BLAST score lower than 200 were discarded. To accurately identify the obtained AMF OTUs, the representative sequence for each OTU was also queried against the MaarjAM database (Öpik *et al.*, 2010; accessed April 13, 2016), a database that aims to provide a quality-controlled repository for published sequence data from Glomeromycota.

5.3.5 Data analysis and statistics

To assess the adequacy of the sampling effort, rarefaction curves were made in MOTHUR (Schloss *et al.*, 2009) for all 34 vineyards, and for all conventional and organic vineyards separately, using a re-sampling without replacement approach. AMF richness was determined as the number of AMF OTUs present in a sample. AMF diversity was approximated by the Shannon diversity index (H) and was calculated using the 'summary.single' command in MOTHUR. Shannon diversity was exponentially transformed (Exp(H)). After this conversion, AMF diversity is measured in units of number of OTUs and the variable behaves linear, in contrast to the non-transformed Shannon diversity (see Jost (2006) for details). Subsequently, a set of spatial predictors were calculated from the geographical coordinates of the vineyards by principle coordinates of neighbor matrices (PCNM), using the 'pcnm' function of the R-package Vegan (Borcard & Legendre, 2002; Borcard *et al.*, 2004; Oksanen *et al.*, 2013). Next, we explored whether conventionally and organically managed vineyards differed in soil chemical composition using linear mixed models in SPSS 22.0 (SPSS Inc., Chicago, IL), with the soil variables as the dependent variables, and management as the fixed factor. Because five samples were taken within a vineyard, we included 'vineyard' as a random factor in the mixed model to account for pseudoreplication. Next, we used linear mixed models to test for relationships between AMF diversity measures, soil chemical variables, management and the spatial PCNM variables. To account for sequencing depth and pseudoreplication, 'sequencing depth' (covariate) and 'vineyard' (random factor) were again included in the model. Subsequently, we build the mixed model with forward selection using the chemical soil variables (pH, N, P, Organic carbon and Cu), management type and the PCNM variables as predictors.

To test for relationships between AMF community composition (i.e. presence/absence of certain OTUs in the AMF community), soil chemical variables, management type and geography, we used unconstrained and constrained ordination. For the unconstrained ordination approach, we performed a non-metrical multidimensional scaling (NMDS) on the sample * OTU matrix, using Bray-Curtis distances based on presence/absence data (R- package Vegan, Oksanen *et al.*, 2013). Subsequently, soil chemical variables, management type and PCNM variables were fitted onto the ordination and tested for significance based on a permutation test with 1000 iterations, using the function 'envfit' (Vegan package). For the constrained ordination approach, canonical redundancy

analysis (RDA) was performed using the R-package Vegan with the soil chemical variables, management type and PCNM as explanatory variables (Oksanen *et al.*, 2013). Note that RDA not only uses the presence/absence data, but also models the abundance of the OTUs. To determine the explanatory variables that significantly explained variation in the AMF communities, forward selection (999 Monte Carlo permutations, $\alpha < 0.05$) was used (R package Packfor).

To detect OTUs specific for organic and conventional vineyards, we performed an indicator species analysis in PC-ORD 6 (Dufrêne & Legendre, 1997; McCune & Mefford, 2006). This analysis calculates an indicator value based on fidelity and relative abundance of an OTU in relation to management type. By definition, an indicator value of 100 (perfect indicator OTU) implies that the presence of a given OTU identifies the management type without error. The obtained indicator values were tested for significance using a Monte Carlo randomization test with 1000 permutations.

Finally, to test whether AMF communities showed a nested structure, i.e. to test whether AMF OTUs detected in OTU-poor vineyard are a subset of the OTUs found in OTU-rich vineyards, two different measures were used to estimate the degree of nestedness. First, a formal nestedness analysis was performed using BINMATNEST (Rodriguez-Girones & Santamaria, 2006). The program calculates the matrix temperature, a measure of nestedness varying between 0° (perfectly nested) and 100° (perfectly non-nested). The significance of nestedness was tested using default input parameters and null model 3. Almeida-Neto *et al.* (2008) demonstrated that matrix temperature may be sensitive to both matrix size and shape, and designed a new metric for nestedness analysis to overcome these flaws. This metric is based on overlap and decreasing fill (NODF) and was calculated using the software package ANINHADO (Guimarães & Guimarães, 2006). To test the significance of nestedness, two different randomization models were used. In the first model (ER) presences are randomly assigned to any cell within the matrix. In the second model (CE) the probability of each cell being occupied depends on the number of presences in the row and column (Almeida-Neto *et al.*, 2008). The CE model allows us to test for statistical significance, given that some vineyards have higher diversity and some taxa are more common than others. In order to assess the relation between the nestedness of the AMF communities, management and soil chemical variables, a Spearman rank correlation coefficient was calculated between the position of the vineyards in the maximally stacked matrix and the soil chemical variables. A Mann-Whitney U test was performed to test for a significant difference in position of the vineyards in the maximally stacked matrix between both management types.

5.4 Results

5.4.1 Pyrosequencing

For all 170 samples together, pyrosequencing resulted in a total of 450 334 filtered reads, with a minimal length of 225 bp and containing the correct barcode and primer sequence. A BLAST search against GenBank revealed the presence of 129 782 (28.8 %) Glomeromycota reads, ranging from 8 to 3969, and an average of 763 AMF reads per sample.

5.4.2 AMF diversity

In total, 123 AMF OTUs were detected. The majority of OTUs belonged to the Glomeraceae (72.4 %, 89 OTUs, 119 472 sequences) and Claroideoglomeraceae (15.4 %, 19 OTUs, 9 443 sequences), whereas only a few OTUs belonged to the Gigasporaceae (5.7 %, 7 OTU, 406 sequences), Diversisporaceae (2.4 %, 3 OTU, 143 sequences), Acaulosporaceae (1.6 %, 2 OTU, 20 sequences), Paraglomeraceae (1.6 %, 2 OTU, 291 sequences) and Archaeosporaceae (0.8 %, 1 OTU, 7 sequences) (Appendix Table D2). The rarefaction curves tended to saturate for almost all vineyards (Appendix Fig. D2), and AMF richness ranged from 16 to 62 OTUs per vineyard (Appendix Table D1). In total, 119 OTUs were observed in the organic vineyards compared to 112 OTUs in the conventional vineyards (Fig. 5.1).

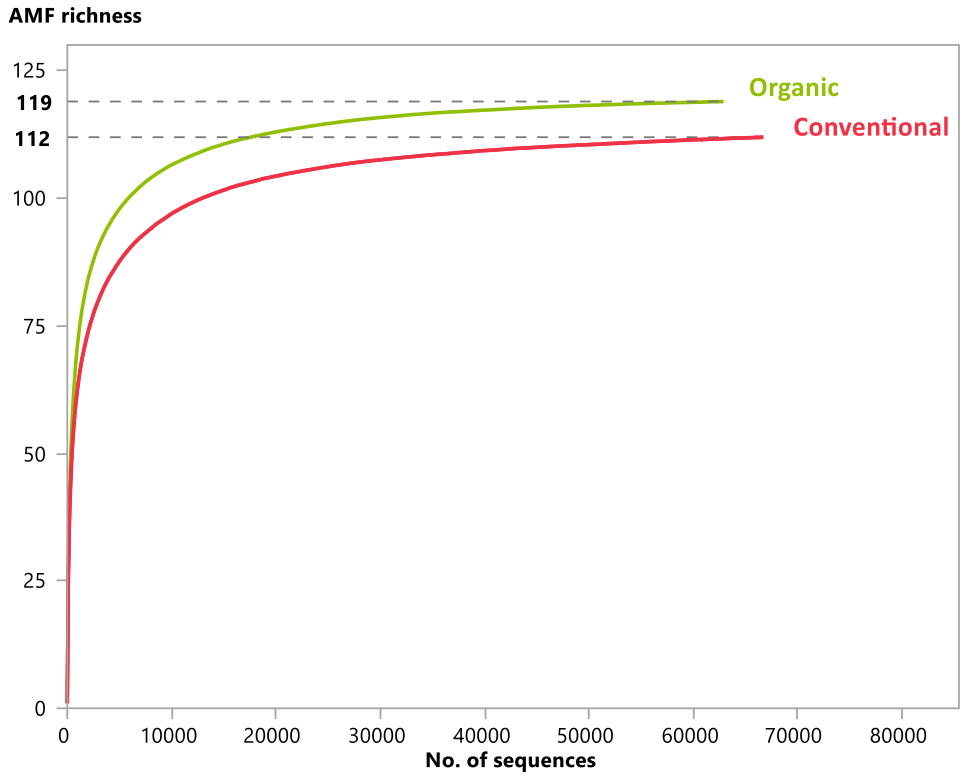


Figure 5.1 Rarefaction curves of AMF richness per management type.

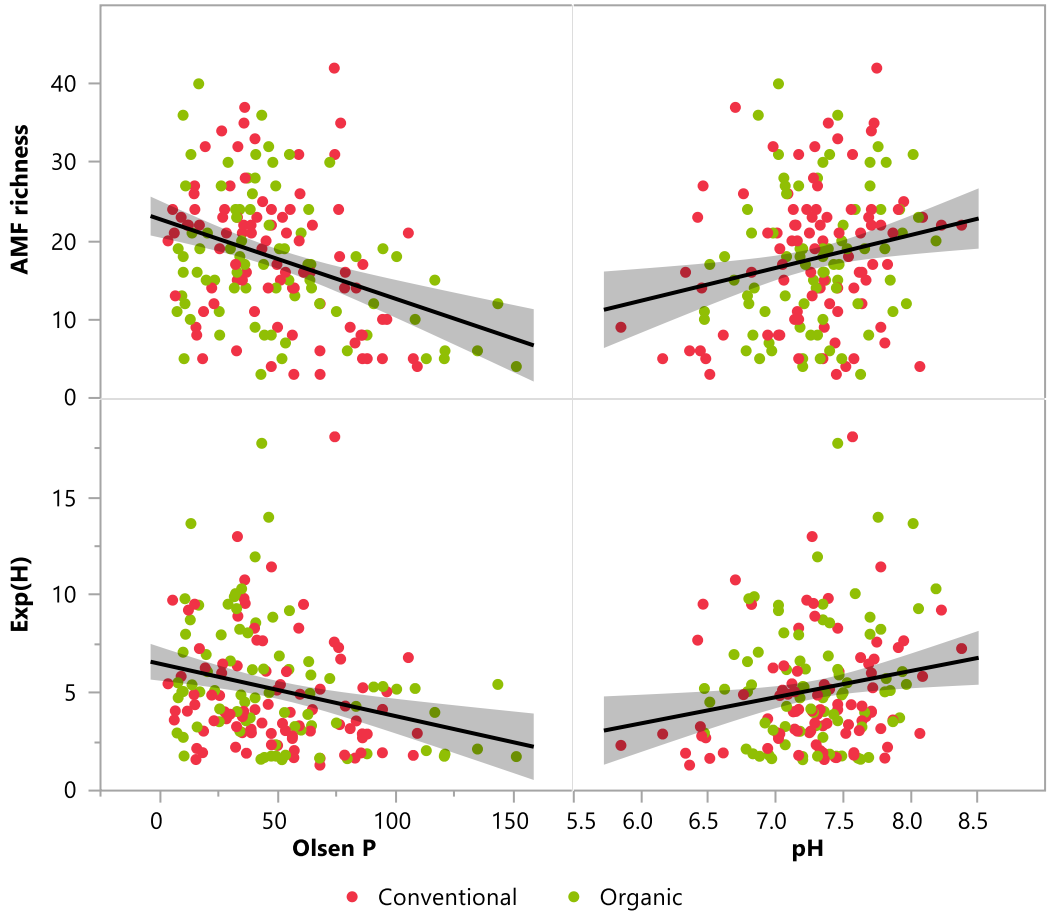


Figure 5.2 Relationship between AMF diversity measures, soil Olsen P and pH. Lines represent marginal models as calculated in the linear mixed model (see Table 5.2).

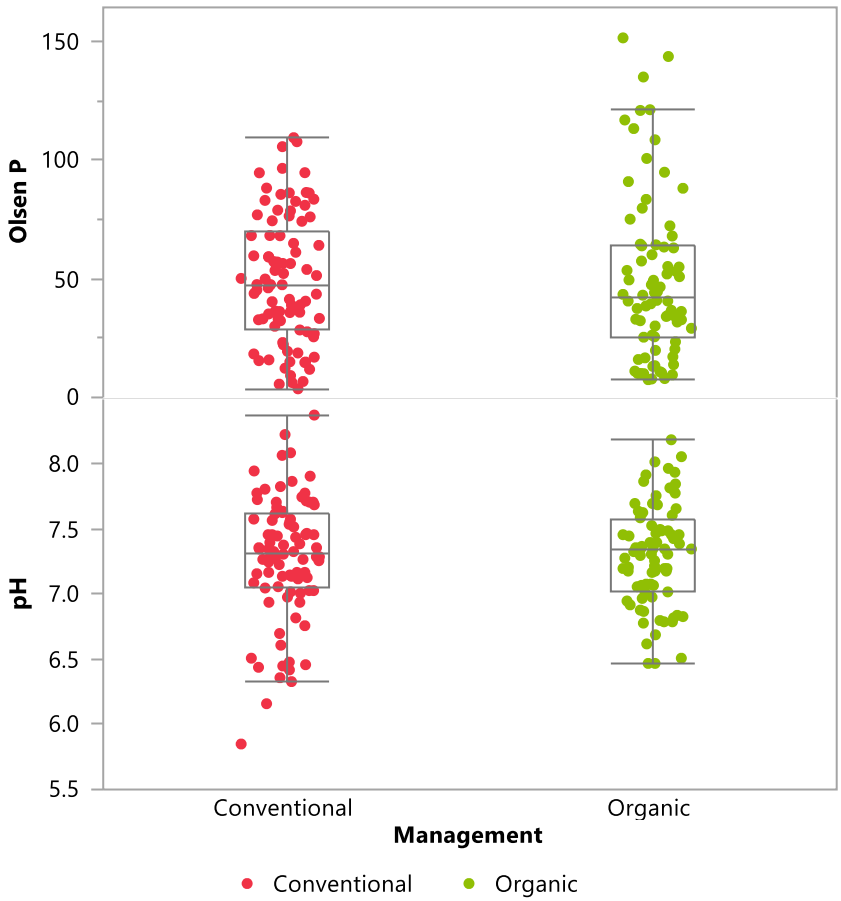


Figure 5.3 The soil chemical variables selected in the forward selection procedure of the mixed model to test for relationships between soil variables and AMF diversity measures, i.e. Olsen P and pH, did not differ between management types. Box plots show 25, 50 and 75 percentiles, and outliers.

The mixed model to test whether the soil chemical variables differed between management types revealed no significant differences (Table 5.1). The mixed model with forward selection revealed Olsen P and pH as the only variables significantly related to AMF richness and Exp(H) (Table 5.2) (Fig. 2 and 3). Copper concentration, management type and PCNM variables, however, were not selected in both models (Fig. 5.4).

Table 5.1 Results of the mixed model analysis to test for differences in soil chemical variables between management types. To account for pseudoreplication, 'vineyard' was included as a random factor. Soil N, Olsen P and Cu are expressed in mg/kg soil.

	Conventional Mean (S.E.)	Organic Mean (S.E.)	*F*	*P*
pH	7.28 (0.064)	7.31 (0.068)	0.046	0.832
Soil N	14.67 (1.61)	16.82 (1.71)	0.834	0.368
Olsen P	49.17 (6.66)	49.33 (7.07)	<0.001	0.988
Organic carbon	0.042 (0.0053)	0.057 (0.0056)	3.738	0.063
Cu	22.59 (2.96)	20.40 (3.13)	0.256	0.616

Table 5.2 Results of the mixed models to test for relationships between AMF diversity measures, soil chemical variables and management. To account for pseudoreplication, 'vineyard' was included as a random factor. To prevent bias due to different sequencing depth, 'sequencing depth' was included as a covariate in both models. Soil N, organic carbon, management and the spatial PCNM variables were excluded by forward selection model procedures.

	Richness			Exp(H)		
	Coefficient	*F*	*P*	Coefficient	*F*	*P*
Intercept	-5.205	0.24	0.633	-2.508	0.41	0.521
Sequencing depth	0.002	6.53	0.012	-0.001	9.03	0.003
Olsen P	-0.081	10.86	0.002	-0.0309	13.53	0.001
pH	3.474	5.59	0.019	1.356	6.60	0.011

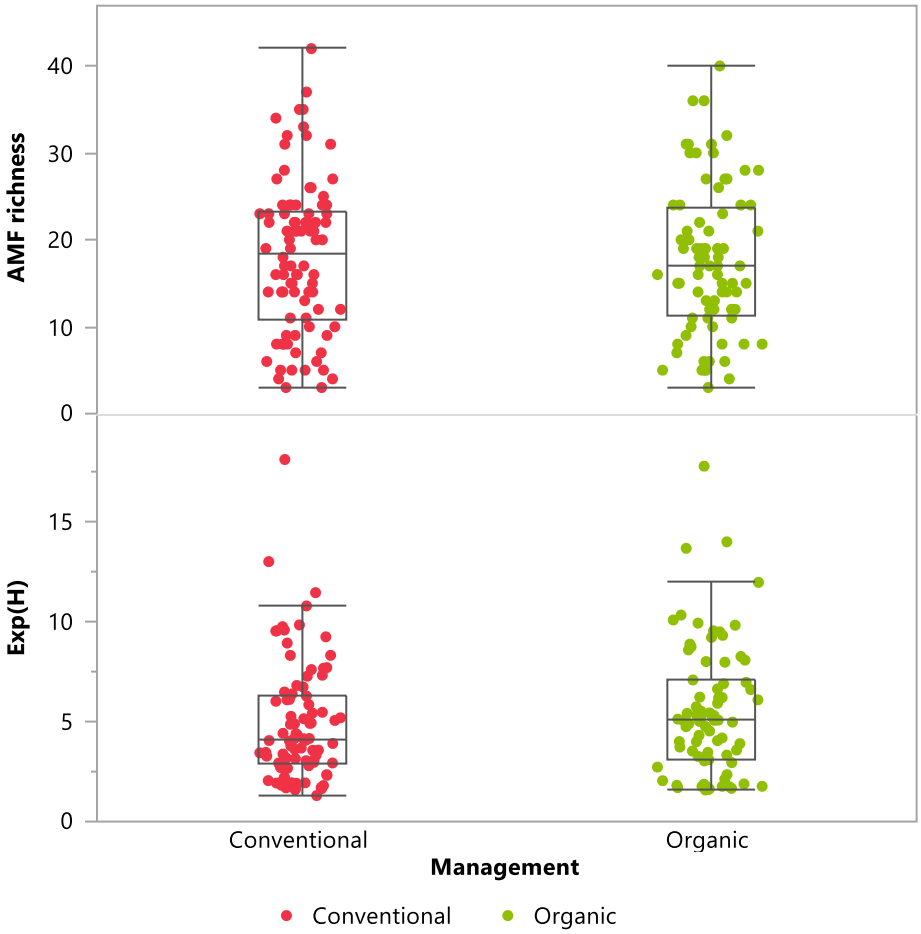


Figure 5.4 No differences in AMF diversity measures were found between conventionally and organically managed vineyards. Box plots show 25, 50 and 75 percentiles, and outliers.

5.4.3 AMF community composition

The NMDS permutation test revealed organic vineyards to harbor significantly different AMF communities as compared to conventional vineyards (Table 5.3, Fig. 5.5). From the soil chemical variables, only Olsen P and pH contributed significantly to AMF community composition (Table 5.3). No significant relations could be found between AMF community composition and nitrogen, organic carbon and copper concentrations in the soil (Table 5.3). PCNM2 was the only variable that was significantly related to AMF community composition (Table 5.3, Appendix Fig. D3).

Table 5.3 Results of the permutation tests of the two dimensional NMDS ordination testing for significant relationships between AMF community composition, soil chemical variables, management and spatial PCNM variables. The results are based on 1000 permutations.

	R^2	*P*
Management	0.021	0.022
pH	0.053	0.017
Soil N	0.005	0.689
Olsen P	0.155	<0.001
Organic carbon	0.004	0.711
Cu	0.010	0.416
PCNM1	0.008	0.465
PCNM2	0.056	0.006
PCNM3	0.001	0.884
PCNM4	0.016	0.265
PCNM5	0.001	0.894
PCNM6	0.002	0.825
PCNM7	0.001	0.898
PCNM8	0.013	0.339

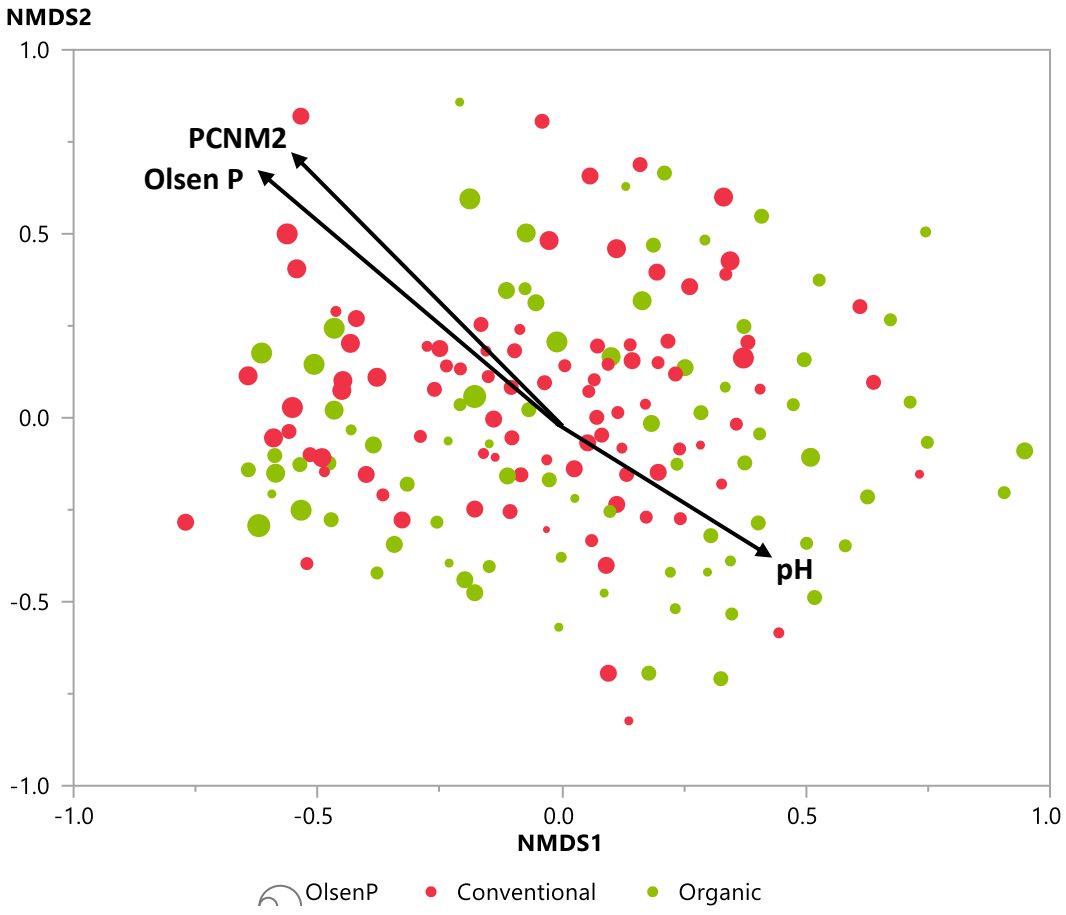


Figure 5.5 NMDS ordination plot of AMF communities from 34 vineyards (5 samples per vineyard). AMF communities between conventional (red) and organic (green) vineyard were significantly different (Table 5.4). Significant relationships between ordination scores, soil chemical and PCNM variables are shown with an arrow, representing the direction of the increasing gradient. Point size represents Olsen P values. Stress value: 19.3.

In agreement with the NMDS permutation test, the forward selection procedure in the direct ordination approach selected Olsen P (R^2_a = 0.031, *P* = 0.019) and PCNM2 (R^2_a = 0.024, *P* = 0.010) as the only explanatory variables. Management could not explain any significant part of the variation in the AMF communities.

5.4.4 Indicator species analysis

The indicator species analysis detected three significant (*P* < 0.05) and important (indicator value > 25) indicative OTUs for the conventional vineyards (OTU_157, OTU_142 and OTU_33). Similarly, one OTU was indicative for the organic vineyards (OTU_45) (Table 5.4). OTU_142 and OTU_45 belonged to the family Glomeraceae (genus Glomus), while OTU_157 and OTU_33 belonged to the Paraglomeraceae and Claroideoglomeraceae, respectively (Table 5.4).

Table 5.4 OTUs indicative for conventional and organic vineyards. Significance levels are obtained by Monte Carlo permutation tests.

OTU ID	Management	No. of reads	Family	Genus	Indicator value	*P*
OTU_157	Conventional	272	Paraglomeraceae	Paraglomus	38.7	<0.001
OTU_142	Conventional	1176	Glomeraceae	Glomus	35.6	0.008
OTU_33	Conventional	3548	Claroideoglomeraceae	Claroideoglomus	33.0	0.002
OTU_45	Organic	1581	Glomeraceae	Glomus	25.8	0.007

5.4.5 Nestedness

The distribution of AMF OTUs showed a nested pattern, as indicated by a matrix temperature of 36.8, which was significantly lower than expected by chance (*P* < 0.001). In agreement, the matrix NODF(Er) was 37.85 (*P* < 0.001) and NODF(Ce) was 44.91 (*P* < 0.001), indicating that the matrix was significantly more nested than expected by chance. The row and column permutated presence/absence vineyard-OTU matrix closest to perfect nestedness is shown in Fig. 5.6. A Mann-Whitney U test revealed no significant difference in position in the stacked minimum temperature matrix between conventional and organic vineyards (*P* = 0.88). In contrast, matrix position significantly correlated with Olsen P (Spearman's rank, r = 0.372, *P* = 0.030) and not with pH, nitrogen, organic carbon and copper in the soil. Therefore, vineyards with higher soil Olsen P harbored increasingly nested AMF communities.

Figure 5.6 Nestedness of AMF communities across 34 vineyards as shown by the row and column permutated presence/absence matrix that is closest to perfect nestedness. Columns represent vineyards (sorted according to their degree of nestedness) and rows are OTUs.

5.5 Discussion

This is the first study characterizing AMF communities in organically and conventionally managed vineyards across a regional scale using a next-generation sequencing approach. Although several studies have shown that organic farming can increase AMF diversity in agricultural settings (e.g. Verbruggen *et al.*, 2010; Van Geel *et al.*, 2015), no differences in AMF diversity between organically and conventionally managed vineyards were found in our study. Instead, plant-available P content of the soil and pH were the only variables significantly related to AMF diversity. These soil variables, however, were similar in both organically and conventionally managed vineyards. Although no chemical fertilizers are allowed in organically managed orchards, still high levels of available P occurred in these vineyards. The two vineyards with the highest available P content in the soil (vineyard 26 and 34) were also both managed organically. Therefore, organic management is no guarantee for high AMF diversity, as organic fertilization can still lead to high available P levels in the soil. This can overrule any beneficial effects of organic management, and consequently still result in a low AMF diversity. This negative relation between AMF diversity and available P content in the soil was also found in the study of Van Geel *et al.* (2015). It is explained by both optimal allocation and increased competition. Phosphorus enrichment through fertilization will reduce plant allocation to roots and consequently the mycorrhizal symbiosis. A reduced plant allocation to AMF will increase competition for plant photosynthates between AMF, and potentially lead to lower AMF diversity (Johnson *et al.*, 2013).

Additionally, a positive correlation between pH and AMF diversity was found. In general, there is a broad agreement that soil acidity can strongly affect soil microbial communities (Fierer & Jackson, 2006; Lauber *et al.*, 2008; Rousk *et al.*, 2010). Jansa *et al.* (2014) also showed that soil acidity was one of the most important drivers of AMF communities in Swiss agricultural soils. Moreover, soil acidity strongly affected AMF communities in the roots of Arabica coffee (De Beenhouwer *et al.*, 2015). Therefore, our results agree with previous studies. It is possible that N enrichment through fertilization lowered pH, as N enrichment can acidify the soil (Vitousek *et al.*, 1997). No effect of soil N on AMF communities, however, was observed. Nitrogen mobilizes easily in the soil, especially under humid conditions. Therefore, effects of N on AMF communities may be difficult to measure.

In agreement with our diversity analysis, the unconstrained and constrained ordination approach revealed that the available P content in the soil was significantly related to AMF community composition. In contrary to the unconstrained ordination, pH was not selected in the constrained ordination approach. Instead, the second spatial predictor (PCNM2) significantly contributed to AMF community composition. PCNM2 separates the vineyard according to their longitude and correlates with unmeasured environmental variables such as soil texture. Subsequently the smaller effect of pH could not further explain any variation in community composition. Although organic farming did not affect AMF diversity in vineyards, AMF communities significantly differed between conventionally and organically managed vineyards in the unconstrained ordination approach. In the constrained ordination, however, management did not explain any significant variation in

community composition, indicating that management type could explain very little variation in AMF community composition. Although the effect of management type on AMF communities may be small, the indicator species analysis detected three indicative OTUs for the conventional vineyards, i.e. OTU_157, OTU_142 and OTU_33. In the MaarjAM database (Öpik *et al.*, 2010), these indicative OTUs were identified as virtual taxa VTX00281, VTX00143 and VTX00278. These virtual taxa have been reported in several anthropogenic systems in the roots of several crops. VTX00281, for example, was observed in a peach orchard and a maize field (Alguacil *et al.*, 2011; Sasvari *et al.*, 2011). Also VTX00143 was found in the soil of arable fields (Daniell *et al.*, 2001). Moreover, VTX00278 was detected in the roots of grapevine (Schreiner & Mihara, 2009). For the organically managed vineyards, the indicator species analysis detected one indicative OTU (OTU_45, identified as VTX00212). Just like VTX00278, VTX00212 was found in vineyards before (Schreiner & Mihara, 2009), indicating these taxa may be well adapted to vineyard conditions.

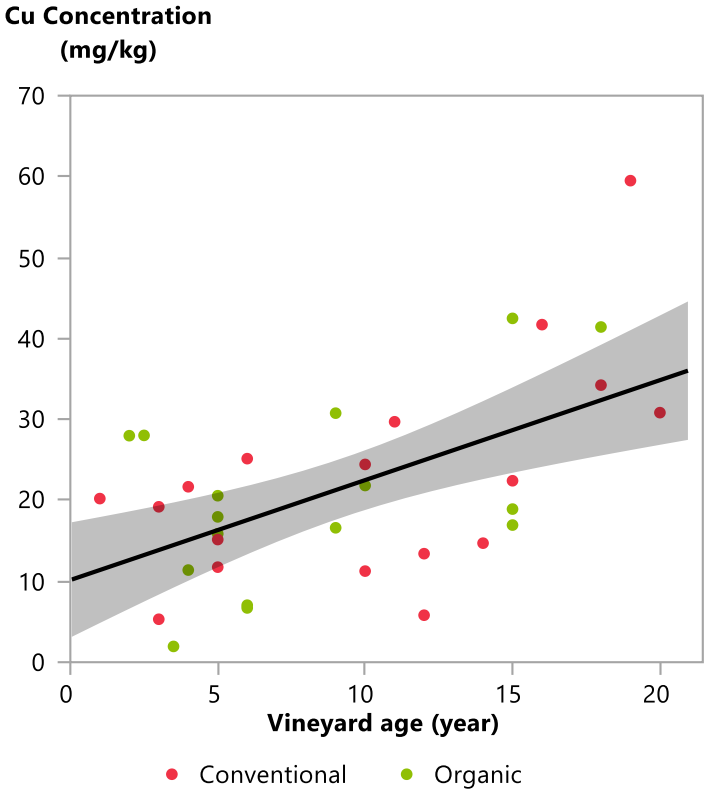


Figure 5.7 The relation between copper concentration in the soil and vineyard age ($P < 0.001$). Older vineyards (> 15 years) show copper concentration above the background level (30 mg/kg).

We found no effects of copper concentration in the soil on AMF diversity and community composition. This can be explained by the relative low copper concentrations measured in the vineyard soils, i.e. the majority of the vineyards (75%) had lower copper concentrations than the background level (30 mg/kg). Also no differences in copper concentration were found between conventionally and organically managed vineyards. However, we observed that copper concentration in the soil increases with vineyard age (Fig. 5.7). Older vineyards (> 15 years) showed copper concentrations above the background level (30 mg/kg), indicating copper is accumulating

in the soil over time. If this trend continues, the copper concentration of a vineyard may reach 100 mg/kg after 73 years of viticulture. Similar observations were made in European vineyards with a long history of copper-based fungicide use (Deluisa *et al.*, 1996; Flores-Vélez *et al.*, 1996).

The AMF communities originating from 34 vineyards across Flanders were organized in a nested pattern. Therefore, poor AMF communities are a subset of the richer AMF communities, indicating a gradual loss of specialist taxa and the occurrence of general taxa. In a total of 170 samples, OTU_2 (identified as VTX00113) occurred in 167 samples. Therefore, this AMF taxa can be considered a generalist. VTX00113 was not only the most frequent taxa in our dataset, but it is also the most abundant taxa in the MaarjAM database. In some entries VTX00113 is identified as *Rhizophagus intraradices*, one of the most widespread mycorrhizal fungus. It has been observed in a wide range of natural and anthropogenic ecosystems, from forests and grasslands to orchards and arable fields. VTX0013 also occurs in high-input agricultural ecosystems, suggesting it tolerates high nutrient levels in the soils (Hijri *et al.*, 2006). Indeed, Sylvia and Schenk (1983) showed that P enrichment did not affect sporulation of *Rhizophagus intraradices*. Additionally, at high plant-available P levels, *Rhizophagus intraradices* can even reduce the growth of citrus trees and therefore become an inferior mutualist (Peng *et al.*, 1993). Furthermore, we found that the degree of nestedness was positively correlated to the plant-available P in the soil. Therefore, higher plant-available P values levels in the soil were related to a gradual loss of specialist taxa and AMF communities dominated by generalists. Consequently, vineyards with high plant-available P (Olsen P > 70 mg P/kg soil) were dominated by generalists. Conversely, vineyards with low plant-available P (Olsen P < 70 mg P/kg soil) harbored more specialist species.

Given that grapevine depends on AMF for nutrient uptake, normal growth and development, and protection against abiotic and biotic stresses, AMF communities will undoubtedly contribute to the microbial terroir of vines (Trouvelot *et al.*, 2015). Although organic farming may increase AMF diversity in agricultural settings, we found that any positive effects of organic management on AMF diversity in vineyards were overruled by soil characteristics. More specifically, plant-available P and pH levels strongly affected AMF diversity, community composition and nestedness in vineyards. Therefore, it is likely that different AMF communities observed in variable soil conditions will result in distinctive grape characteristics. Additionally, a vineyard harboring many AMF specialists will likely result in unique grape characteristics compared to a vineyard dominated by generalists. Vine growers can control plant-available P and pH of their vineyard soils and thus directly influence the AMF communities present in the roots of grapevine and potentially also grape characteristics. Future research should focus on identifying the effect of different AMF communities on the characteristics of grapes, and disentangle them from the effect of changing soil characteristics.

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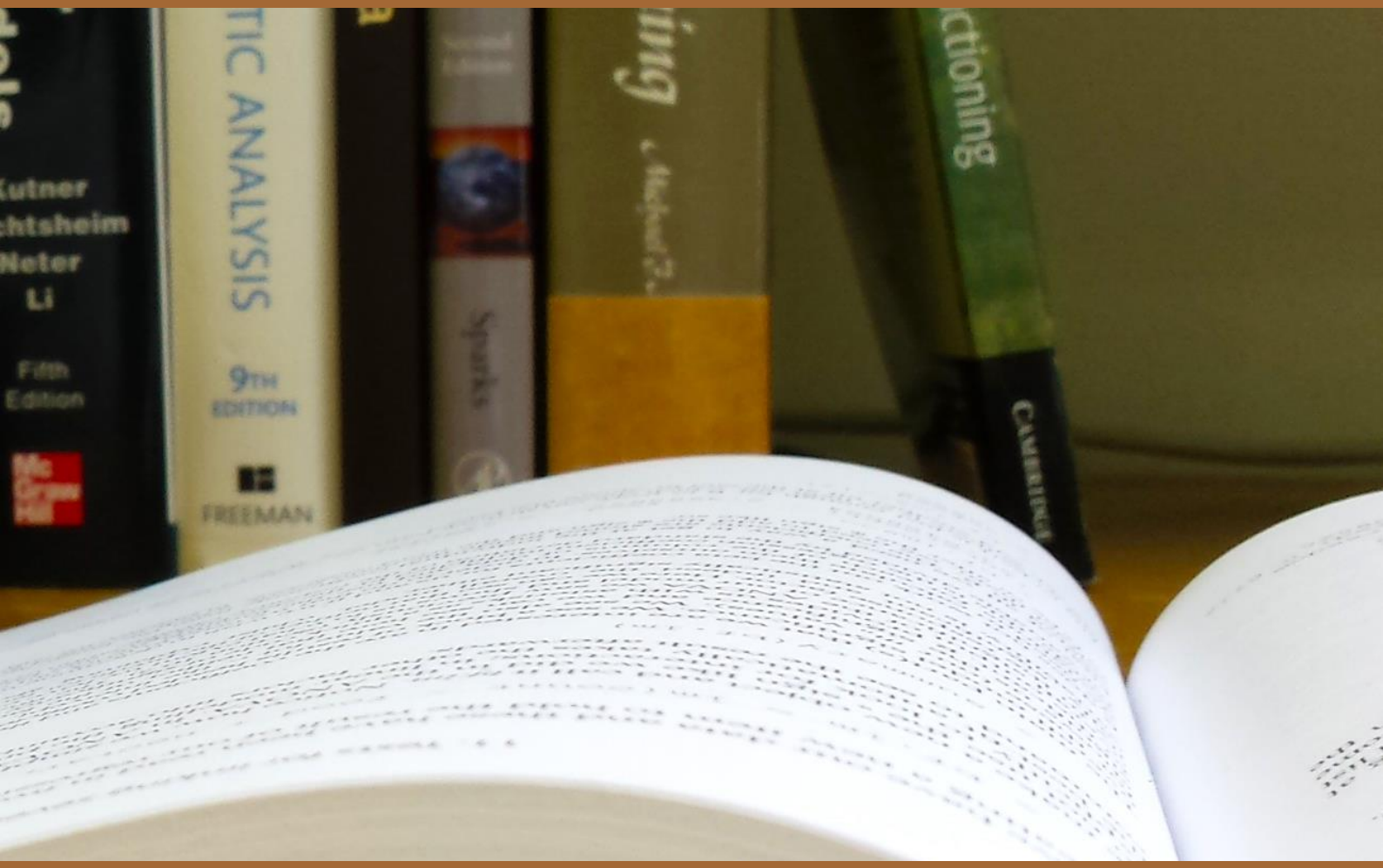
Chapter 6

Crop specific and single species mycorrhizal inoculation is the best approach to improve crop growth in controlled environments



Adapted from:

Van Geel Maarten, De Beenhouwer Matthias, Lievens Bart, Honnay Olivier. 2016. Crop specific and single species mycorrhizal inoculation is the best approach to improve crop growth in controlled environments. *Agronomy for Sustainable Development*. Accepted.

6.1 Summary

Arbuscular mycorrhizal fungi are root symbionts that play a key role in crop growth. A systematic quantitative analysis of the response of crops to arbuscular mycorrhizal inoculation, however, remains to be done. Additionally, little is known regarding the role of mycorrhizal specificity and the diversity of the inoculum on crop growth. Therefore, we collected data from 115 inoculation studies, including 435 experiments. We then used meta-analysis to examine the effect of crop identity, arbuscular mycorrhizal fungus identity, and mycorrhizal diversity on crop biomass increase, following inoculation. Our results show that total crop biomass was on average 34.9% higher in inoculated versus non inoculated plants. We found that specific combinations of arbuscular mycorrhizal fungus genera and host plant families were more beneficial for growth promotion as compared to other combinations. Moreover, a single-species inoculum increased crop growth response on average by 41.2% compared to a multi-species inoculum. Overall, our findings show that a broad range of crops highly benefit from the inoculation with arbuscular mycorrhizal fungi. They also strongly suggest that selecting specific arbuscular mycorrhizal taxa for specific crops is the most promising approach to enhance crop growth. There is no 'one-size-fits-all' arbuscular mycorrhizal fungus. Finally, and at least in stable and controlled environments, inoculation with a single arbuscular mycorrhizal species is more effective, compared to inoculation with a mixture of different arbuscular mycorrhizal taxa. This may be explained by fungi superior in extraradical growth, but less beneficial to the host, that outcompete the more mutualistic fungi. Therefore, it may be beneficial to maintain a high dominance of one beneficial arbuscular mycorrhizal taxon in simplified agricultural systems.

6.2 Introduction

Arbuscular mycorrhizal fungi (Glomeromycota) (AMF) are widespread and obligate plant symbionts known to play a key role in the functioning of agricultural ecosystems and crop productivity (Verbruggen *et al.*, 2010). These fungi establish a symbiosis with the majority of the land plants and generally provide mineral nutrients to the host plant in exchange for plant assimilated carbohydrates (Smith & Read, 2008). Furthermore, they form a large network of hyphae and have a great impact on soil formation and soil aggregation (Wilson *et al.*, 2009). Finally, AMF can provide protection of the host plant against fungal and nematode pathogens (Veresoglou & Rillig, 2012). Overall, AMF have been shown to increase host plant biomass production (Hoeksema *et al.*, 2010), although a systematic analysis on the response of crop species in particular remains to be done. However, whereas the symbiosis is often seen as an unambiguous mutualism where especially the host plants often benefit from the association, AMF have also been reported to be of little benefit to the host plant and even to be parasitic by causing a net carbon cost for the host (Johnson *et al.*, 1997; Graham & Eissenstat, 1998).

Based on morphological characteristics of spores, 244 arbuscular mycorrhizal species have been described so far (Schüssler, 2014). Direct sequencing of fungal ribosomal RNA genes from the soil on the other hand have revealed the existence of 341 virtual arbuscular mycorrhizal taxa so far (operational taxonomic units (OTUs)) (Öpik *et al.*, 2013). Consequently, the number of known arbuscular mycorrhizal species/virtual taxa is very low, compared to the *c.* 200.000 plant species they associate with (Brundrett, 2009), suggesting that arbuscular mycorrhizal specificity to the host plant is very low (Mosse, 1975; McGonigle & Fitter, 1990). Nevertheless, some studies found evidence that co-existing plant species harbor different arbuscular mycorrhizal communities (Vandenkoornhuyse *et al.*, 2003; Torrecillas *et al.*, 2012). Furthermore, specificity in the arbuscular mycorrhizal symbiosis may also occur at a higher level, such as the ecological group (Öpik *et al.*, 2009) or family (Torrecillas *et al.*, 2012) of the host plant.

In addition to the extent of AMF specificity, little is known regarding the role of arbuscular mycorrhizal diversity on host plant growth. In a more general context, there is now convincing evidence that higher species diversity is generally beneficial for a range of ecosystem functions (Cardinale *et al.*, 2012). For example, a higher plant species or pollinator species richness may result in higher biomass production and crop pollination, respectively, either through the sampling effect or through the complementarity effect. The sampling effect refers to having a greater chance of including a very effective species with respect to the ecosystem functioning, whenever there is a higher species diversity (Turnbull *et al.*, 2013). The complementarity effect refers to resource partitioning through functional complementarity, which leads to a more efficient exploitation of resources in the system (Tilman, 1997). Also arbuscular mycorrhizal taxa are known to be functionally different, for example regarding the formation of extra-radical hyphae, colonization rates and their phosphorus foraging strategy (Hart & Reader, 2002). This may result in a more efficient exploitation of soil resources in case of the presence of different arbuscular mycorrhizal

taxa, and a direct fitness benefit for the host plant. For example, compared to colonization by a single arbuscular mycorrhizal fungus, colonization by two AMF with different spatial abilities to acquire soil phosphorus induces a larger growth response in the host plant (Smith *et al.*, 2000). Although van der Heijden *et al.* (1998) already provided insights in the relation between mycorrhizal diversity and ecosystem functioning, their results were based on microcosms and macrocosm mimicking European calcareous grasslands, and are as such difficult to transpose to crops in an agricultural context. Even though there have been many experimental studies testing the growth response of a broad range of crops to single and multiple mycorrhizal inoculations, the results obtained appear to be inconsistent. On the one hand, many studies have shown an increased growth benefit of crops to inoculation with multiple AMF compared to inoculation with a single arbuscular mycorrhizal fungus (e.g. Ortas & Ustuner, 2014; Pellegrino & Bedini, 2014). On the other hand, Hart *et al.* (2013), for example, showed that high fungal diversity in the roots of a plant can facilitate the persistence of low-quality symbionts, resulting in a very limited growth benefit to their host. Consequently, it is still unclear to what extent mycorrhizal diversity can promote growth in the host plant.

Meta-analysis is a statistical technique used to summarize and quantify a selected set of studies (Borenstein *et al.*, 2011). Given the large number of studies, performed with different crops and different AMF, a meta-analysis now allows to integrate their results and provide quantitative answers regarding the outcome of different arbuscular mycorrhizal taxa-host plant combinations, and to what extent mycorrhizal diversity can promote crop growth. More specifically, the objectives of this study were to: (i) quantify the overall growth response of crop species to arbuscular mycorrhizal inoculation; (ii) assess the importance of arbuscular mycorrhizal specificity on the growth response of crop species; and (iii) evaluate to what extent arbuscular mycorrhizal diversity can promote crop growth.

6.3 Materials and methods

6.3.1 Data compilation

Data were compiled based on articles retrieved from a Web of Knowledge search on the 15th of October 2015, using the search string 'arbuscular mycorrhiza* AND inoculat*' (resulting in 1701 articles). Articles were then included in the meta-analysis when they included at least one experiment that met the following criteria: (i) a crop was inoculated with one or more arbuscular mycorrhizal taxa, and the vegetative or generative response (root, shoot or fruit biomass) was compared with the non-mycorrhizal crop (control); (ii) both a measure of variance in plant response and the number of replicates were reported. Furthermore, when multiple phosphorus levels were compared within a study, we only included the 'normal' treatment in our meta-analysis, and not the fertilized treatment.

From each experiment that met these criteria, we retrieved data on plant growth (root, shoot, total and/or fruit biomass), with and without mycorrhizal inoculation. If not reported, standard deviations were calculated from sampling sizes and standard errors. When results were only

reported in figures, the raw data were extracted using GetData Graph Digitizer (version 2.26; http://getdata-graph-digitizer.com). From each experiment, also crop species, AMF taxon (species names were annotated according to Schüssler and Walker (2010)) and inoculum type (single-species *vs.* multi-species inoculum) were recorded.

6.3.2 Statistical analysis

For each experimental comparison between inoculated and control treatments, an effect size for plant biomass response was calculated. We used total plant biomass when available or calculated it as the sum of root and shoot biomass. In the minority of cases where total plant biomass was not available or could not be calculated, we used shoot biomass as a proxy for total plant biomass. We then used the response ratio $Ln(R) = Ln(X_{AMF}/X_c)$ as the effect size, where X_{AMF} and X_c are the mean total biomass values for the inoculated and control treatments, respectively (Hedges *et al.*, 1999). A positive value of Ln(R) indicates a positive effect of AMF inoculation on total plant biomass. A value of Ln(R) = 0 indicates that AMF inoculation had no effect. For each experimental comparison, both Ln(R) and the variance of Ln(R) were calculated using MetaWin v2.1 (Rosenberg *et al.*, 2000). Because only a limited number of experimental comparisons between AMF inoculation and control treatments (66 out of 435, 15 %) reported a measure of fruit biomass, no meaningful analysis was possible on the effect of AMF inoculation on fruit biomass.

We then used general linear models (GLMs) in SPSS 20.0 (SPSS Inc., Chicago, IL, USA) to simultaneously estimate the effects of multiple explanatory variables on total plant biomass to AMF inoculation. Two models were ran with Ln(R) as the response variable, and the following fixed explanatory variables: AMF Genus, Crop Family, AMF Genus x Crop Family (model 1); and Inoculum type (single species or multi-species inoculum) (model 2). The observations with multiple AMF taxa were omitted in model 1. The Bayesian information criterion (BIC) was used to select the most parsimonious model out of a suite of reduced models compared with the full model (i.e. with the lowest BIC). In all cases, we used the full model which had the lowest BIC. The high number of crop species and arbuscular mycorrhizal taxa in our dataset did not allow to conduct a meaningful analysis at the species level. It is normal conduct in meta-analysis to give higher weight to more accurate effect sizes (Borenstein *et al.*, 2011). Therefore, a weight factor (1/variance of the effect size) was included in all models.

When a study reported more than one experiment, these experiments were included as separate data records. To test whether this approach may have led to an overrepresentation of the effect of studies that included a high number of experiments, we randomly chose one observation from each study and conducted the same analysis (He & Dijkstra, 2014). The mean effect sizes that were calculated this way were similar to the effect sizes based on the whole dataset, suggesting that overrepresentation did not occur (He & Dijkstra, 2014). Finally, the presence of a publication bias was tested using scatter plots of effect size *versus* their variance and the sample size of each experiment. No patterns indicative of publication bias could be discovered (Appendix E2).

6.4 Results and discussion

In total, 115 publications met our criteria, resulting in 435 experimental comparisons between AMF inoculation and control treatments (Appendix E1). Both models showed an overall positive response of total biomass to inoculation with AMF. Our first model estimated the overall response ratio of total biomass at 0.290 (95% CI: 0.133 to 0.448), the second model at 0.307 (95% CI: 0.248 to 0.366). Although our first model found no overall significant main effect of AMF Genus, the 95% confidence intervals of the mean effect sizes for the genera *Glomus* and *Funneliformis* did not include zero, indicating a positive growth response to inoculation with AMF (Fig. 6.1a). Similarly, although there was no main effect of Crop Family, the crop families Cucurbitaceae and Poaceae showed the strongest positive response to AMF inoculation and the 95% confidence intervals of the effect sizes did not include zero (Fig. 6.1a). Whereas no main effects of AMF Genus and Crop Family on total plant biomass were found, the significant interaction between AMF Genus and Crop Family indicated that the effect of AMF Genus is dependent on Crop Family (Table 6.1), and that the main effects discussed above have to be interpreted in this context. Especially the symbiosis between AMF from the genera *Funneliformis* or *Glomus* and crops from the Cucurbitaceae, and AMF from the genus *Rhizophagus* and crops from the Rubiaceae resulted in a strong plant growth response (Fig. 6.1a). On the contrary, the symbiosis between AMF from the genera *Funneliformis* or *Glomus* and crop plants from the family Solanaceae did not result in a positive growth response (95% confidence intervals did overlap with zero). Also, the symbiosis between AMF from the genera *Rhizophagus* and crop plants from the family Cucurbitaceae did not result in a positive growth response.

Table 6.1 Results from the general linear model analysis relating the effect size for total plant biomass to the genus of the inoculated AMF and the crop family (model 1), and to the inoculum type (model 2).

Explanatory variables	df	*F*	*P*
model 1			
Intercept	1	3.815	0.052
AMF Genus	9	0.469	0.895
Crop Family	13	1.451	0.135
AMF Genus * Crop Family	29	1.690	0.017
model 2			
Intercept	1	104.265	<0.001
Inoculum Type	1	33.545	<0.001

Whereas AMF were originally considered to be non-specific (Mosse, 1975; McGonigle & Fitter, 1990), more recent studies demonstrated an affinity of certain arbuscular mycorrhizal taxa for specific plant species or cultivars (e.g. Vestberg, 1992; Vandenkoornhuyse *et al.*, 2003; Torrecillas *et al.*, 2012). Our results demonstrate that specific combinations of AMF genera and host plant families are more efficient for growth promotion of crops as compared to others. We showed that arbuscular mycorrhizal specificity may also translate into differing growth responses of crops. Insufficient available data in the literature did not allow us to systematically evaluate specificity at

the species level. Nevertheless, when the most frequently occurring arbuscular mycorrhizal species in our dataset was considered, *Funneliformis mosseae* (n = 104), still large differences in plant growth were found between crop species ($P < 0.001$). Cucumber, for example, showed a significantly higher growth response compared to Cotton, Maize, Tobacco or Tomato when inoculated with *Funneliformis mosseae*, suggesting a trend of arbuscular mycorrhizal specificity at the species level as well.

Our second model resulted in a significant effect of Inoculum Type on the effect size for total plant biomass (Table 6.1). Although both inoculum types (single species vs. multi-species inoculum) resulted in a positive growth response, the single-species inoculum had a mean growth response of 0.481 (95% CI: 0.433 to 0.528), compared to only 0.133 (95% CI: 0.025 to 0.241) for the multi-species inoculum (Fig. 6.1b). These results were somewhat counter-intuitive as it was expected that sampling and complementarity effects would result in higher plant growth following inoculation with multiple arbuscular mycorrhizal taxa. On the contrary, our analysis indicates that crop plants that could have been colonized by multiple arbuscular mycorrhizal taxa perform less, compared to crop plants colonized by a single arbuscular mycorrhizal taxon. These findings resemble those of Veresoglou and Rillig (2012) who found that a host plant colonized by multiple arbuscular mycorrhizal taxa suppresses fungal pathogens less efficiently than when colonized by a single arbuscular mycorrhizal species.

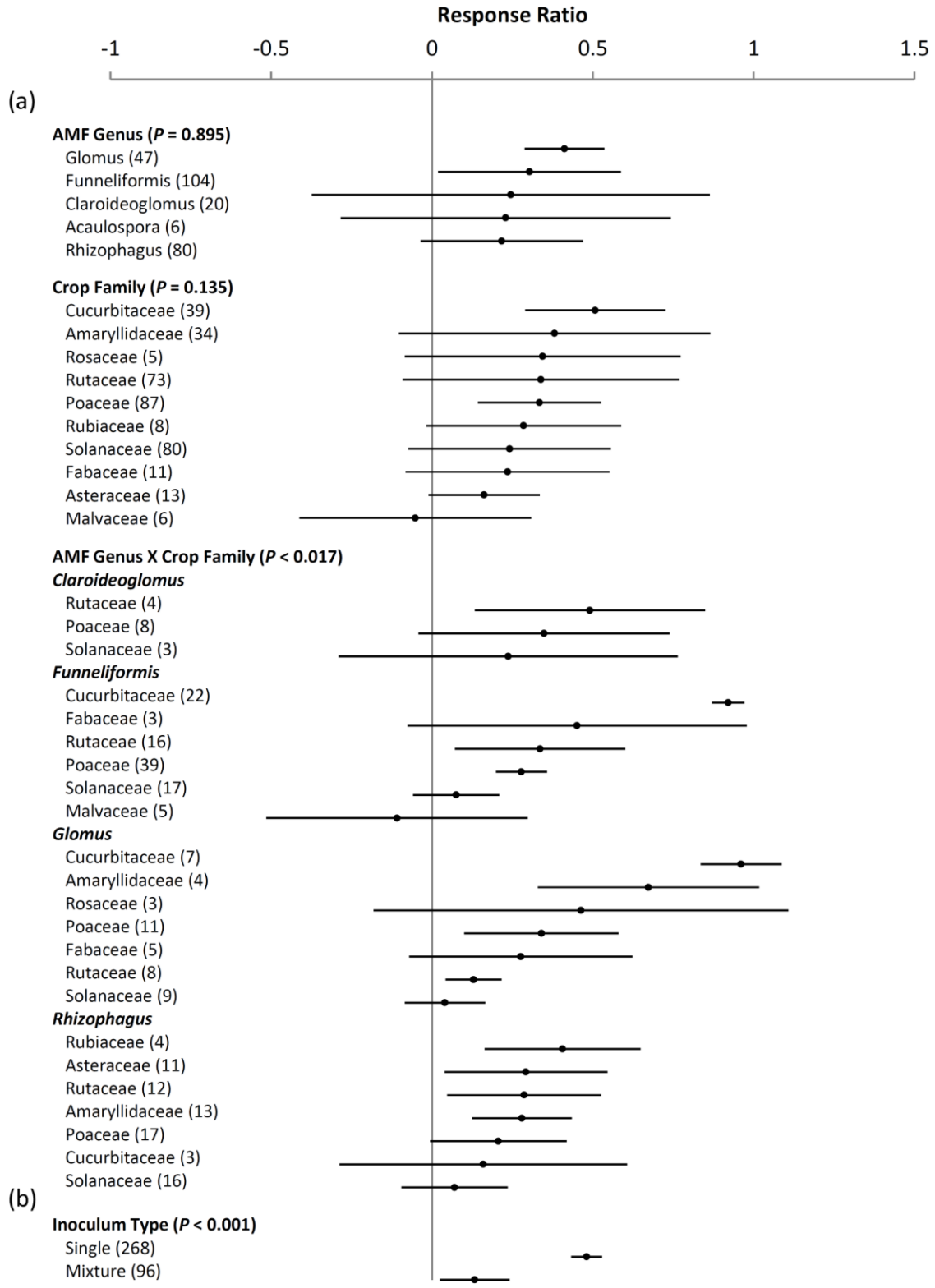


Figure 6.1 The estimated marginal means (± 95% confidence intervals) of the general linear model analyses relating the effect size for the total plant biomass to the identity of the inoculated AMF and the identity of the crop (a) and to the inoculum type (b). The effect of arbuscular mycorrhizal inoculation was considered statistically significant if the 95% confidence intervals of the mean effect size did not overlap with zero. A positive response ratio (Ln(R)) indicates a positive effect of arbuscular mycorrhizal inoculation on total plant biomass. P-values reported are those obtained in both general linear model analyses (Table 6.1). The number of observations in each group is shown in parentheses. Groups with less than 3 observations are not shown.

van der Heijden *et al.* (1998) found that increasing the arbuscular mycorrhizal diversity in artificial mesocosms led to increased plant productivity. The proposed mechanism for this effect, however, was based on variable plant growth responses of different arbuscular mycorrhiza-host plant combinations. In our model 1, we also demonstrated variable growth responses of different arbuscular mycorrhiza-host plant combinations, and therefore, our results, based on growth responses of individual crops, are consistent with van der Heijden *et al.* (1998). Additionally, the lower effect on the growth response of crops of the multi-species compared to the single species inocula can be explained by the nature of the studies included in our meta-analysis, which were mainly short-term inoculation studies performed in controlled artificial environments. Indeed, a higher magnitude and stability of ecosystem functions associated with diversity are likely most evident under changing and/or heterogeneous conditions that crops experience in the field. In plant communities, the stabilizing effect of diversity on ecosystem properties can be explained by temporal complementarity between species (Loreau & de Mazancourt, 2013). In stable controlled experiments, however, temporal complementarity can hardly play any role.

In these stable controlled environments, the lower effect of arbuscular mycorrhizal diversity on individual host plants may be explained by competition among arbuscular mycorrhizal taxa, with AMF superior in extraradical growth, but less beneficial to the host plant, outcompeting more mutualistic AMF (Werner & Kiers, 2015b). Hart *et al.* (2013), moreover, have shown that diverse arbuscular mycorrhizal communities on *Plantago lanceolata* can support the persistence of a less-beneficial symbiont. Complementary, also priority effects, *i.e.* the impact of species arrival on subsequent community development, may negatively affect host plant growth following inoculation with different arbuscular mycorrhizal taxa. Werner and Kiers (2015a) argued against space limitation as the mechanism allowing priority effects to emerge in the arbuscular mycorrhizal fungal system. Instead, their data supported an active down-regulation of specific mycorrhizal partners by the host. Such systemic suppression of AMF by the host has also been observed in split-root experiments in which an established arbuscular mycorrhizal symbiosis suppressed subsequent colonization by different arbuscular mycorrhizal species in the second side of the split-root system (Vierheilig *et al.*, 2000). Although arbuscular mycorrhizal species were inoculated simultaneously in all studies in our dataset, certain taxa may be more efficient colonizers and may consequently inhibit colonization by AMF that are more beneficial to the host plant.

As said, our meta-analysis is based on results from short-term inoculation studies performed in controlled artificial environments. Plants are grown separately in a soil that has been kept moist, autoclaved, mixed with sand and placed in plastic pots. The soil is inoculated with a single arbuscular mycorrhizal fungus that may not naturally interact with the host. Also, the host is not exposed to naturally occurring arbuscular mycorrhiza, pathogens, or plants. Therefore, caution is required before extrapolating our results to natural settings with complex community interactions and changing environmental conditions (Lekberg & Koide, 2014).

We used a multi-factor model with two categorical predictors to assess the role of different arbuscular mycorrhizal taxa and crop families on the response of crops to mycorrhizal inoculation.

Because data availability was dependent on what was found in the literature, some combinations of levels of predictor variable occurred more than others, generating incomplete orthogonality between the explanatory variables of our model 1. In such an unbalanced design, the fitted parameter values may be different from the observed parameter values. Although some small differences between fitted and observed parameter values occurred in our dataset, the data from the observed parameter values generally supported the same conclusions.

6.5 Conclusion

On the one hand, our results confirm that a broad range of crop plants can benefit from the inoculation with AMF, and that there is high potential for additional growth. On the other hand, our results suggest that specific arbuscular mycorrhizal taxa-host plant combinations enhance the growth of a crop, and that, at least in stable and controlled environments, inoculation with a single arbuscular mycorrhizal species may be more beneficial to crop species, compared to inoculation with a mixture of different arbuscular mycorrhizal taxa. In other words, our results indicate that there is no 'one-size-fits-all' arbuscular mycorrhizal fungus, and suggest that there may be potential benefits in maintaining high dominance of one very beneficial arbuscular mycorrhizal taxon. Yet, caution is required when extrapolating our results to natural field conditions with more complex community interactions and variable environmental conditions. Further research should focus on finding the particular arbuscular mycorrhizal taxon-host plant combination that will maximize growth response, both *ex situ* and under field conditions. Finally, we encourage researchers conducting arbuscular mycorrhizal inoculation experiments to report also on the marketable part of the plant, and not only on the root and shoot biomass.

Chapter 7

General discussion

7.1 Main results

In this PhD dissertation, we presented research on diversity, community composition and function of AMF in agricultural ecosystems. The general aim of this research was to gain a better understanding of how environmental factors, such as site geography, soil properties and management, influence the diversity and community composition of AMF in agricultural systems. Additionally, we aimed to assess the role of AMF specificity and diversity on crop performance. In this research, we performed a meta-analysis, two observational studies, a field experiment and compared methods to characterize AMF communities.

In chapter two, we evaluated six primer pairs targeting the nuclear rRNA operon for characterization of AMF communities using 454 pyrosequencing. We showed that primer pairs AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 outperformed the other tested primer pairs in terms of number of Glomeromycota reads (AMF specificity and coverage). Additionally, these primer pairs were found to have no or only few mismatches to AMF sequences and were able to consistently describe AMF communities from apple roots. However, whereas most high-quality AMF sequences were obtained for AMV4.5NF-AMDGR, our results also suggest that this primer pair favored amplification of Glomeraceae sequences at the expense of Ambisporaceae, Claroideoglomeraceae and Paraglomeraceae sequences.

In chapter three, we studied the response of AMF in the roots of cultivated apple trees with increasing orchard management intensity across a regional scale. We characterized the diversity present in the apple roots using 454-pyrosequencing and investigated which environmental factors drive AMF diversity and community composition. We showed that soil characteristics and management type, rather than the geographical location of the orchards, shape AMF communities in the roots of apple trees. Particularly, plant-available P content of the soil was negatively correlated to AMF diversity. Finally, the degree of nestedness of the AMF communities was related to plant-available P and N content of the soil, indicating at a progressive loss of AMF taxa with increasing fertilization.

In chapter four, we experimentally tested whether different amounts and forms of phosphorus fertilizer affected AMF diversity and community composition associated with the roots of apple trees. We found that the slow-release fertilizer treatments showed significantly higher AMF richness and differed in community composition compared to the inorganic fertilizer treatments. The distribution of AMF OTUs showed a significantly nested pattern. Additionally, AMF communities in the inorganic fertilizer treatments were a subset of the communities in the slow-release fertilizer treatments.

In chapter five, we focused on the AMF diversity present in vine roots and investigated which environmental factors drive AMF diversity and community composition. We found no differences in AMF diversity between organically and conventionally managed vineyard. Instead, plant-available P content of the soil and pH, which were similar in both organically and conventionally managed vineyards, were the only variables significantly related to AMF diversity. In agreement

with our diversity analysis, we found that available P content in the soil was also significantly related to AMF community composition. The effect of management type on AMF community composition, however, was unclear, indicating management type could explain very little variation in AMF community composition. Although we found no effects of copper concentration in the soil on AMF diversity and community composition, we observed that older vineyards (> 15 years) showed copper concentrations above the background level (30 mg/kg). Additionally, we found that the AMF communities occurring in the roots of grapevine were organized in a nested pattern. The degree of nestedness was positively correlated to the plant-available P in the soil and negatively correlated to soil acidity.

In chapter six, we applied meta-analytical techniques on the literature reporting on the response of crop plants to examine the effect of crop identity, AMF identity, and AMF diversity on crop biomass increase, following inoculation. Our results showed that total crop biomass was on average 34.9% higher in inoculated versus non inoculated plants. We found that specific combinations of AMF genera and host plant families were more beneficial for growth promotion as compared to other combinations. Moreover, a single-species inoculum increased crop growth response on average by 41.2% compared to a multi-species inoculum.

7.2 The effect of soil characteristics on AMF communities

In both our observational studies (chapter 3 and 5) and our field experiment, the available P in the soil was the most important factor that influenced AMF diversity and community composition. Verbruggen *et al.* (2012), who studied AMF communities in agricultural fields in the Netherlands using T-RFLP analysis, also found available P to be the most important soil variable related to AMF richness in the roots of maize and potato. Lower AMF diversity under a high plant-available P content has been explained by both optimal resource allocation and biotic interactions (Johnson et al. 2013). Optimal allocation predicts that plants should allocate biomass to structures that best garner the most limiting resources. Consequently, the enrichment by inorganic P through fertilization will reduce plant allocation to roots and the mycorrhizal symbiosis, therefore increasing the competition for carbohydrates between AMF species, potentially leading to a lower richness. Indeed, competition can be sufficiently strong to exclude some AMF species from host roots (Hepper et al. 1988). Likewise, we also found very different AMF communities in orchards and vineyards with a high and low plant-available P content of the soil (chapter 3 and 5). In agreement, Treseder (2004) showed using a meta-analysis approach that P additions negatively influence AMF colonization in a wide range of habitats. However, responses to N addition were less consistent compared to responses to P. Indeed, in our research no effects of soil N on AMF diversity or community composition were observed (chapter 3 and 5). Nitrogen mobilizes easily in the soil, especially under humid conditions. Therefore, effects of N on AMF communities may be difficult to measure.

There is a broad agreement that also soil acidity can strongly affect soil microbial communities (Fierer & Jackson, 2006; Lauber et al., 2008; Rousk et al., 2010). Soil acidity also affected AMF

communities in Swiss agricultural soils and in the roots of Arabica coffee (Jansa *et al.*, 2014; De Beenhouwer *et al.*, 2015). In our research, however, AMF diversity and community composition was only related to soil acidity in vineyards (chapter 5), and not in apple orchards (chapter 3). In addition, the effect of soil acidity on AMF diversity and community composition in vineyards was considerably smaller compared to the effect of available P levels in the soil. In apple orchards, however, we collected only one pooled sample per orchard, while in vineyards we took a soil sample near each sampled plant, thus increasing the sample size. Therefore, this may have increased the statistical power of our AMF analysis in vineyards (chapter 5), and thus the ability to detect the smaller effect of pH on AMF diversity and community composition. Indeed, when we averaged the data from chapter five per vineyard and subsequently analyzed the data on the site-level, only Olsen P ($P < 0.001$) and not pH ($P = 0.54$) was selected as a significant variable contributing to AMF richness.

The AMF communities detected in chapter three to five were organized in a nested pattern. Therefore, poorer AMF communities are subset of the richer communities, indicating a gradual loss of specialist taxa and the occurrence of general taxa. Indeed, we found that the AMF taxon identified as VTX00113 in the MaarjAM database was the most common AMF taxon in both our observational studies and our field experiment, occurring in almost all samples. This generalist species was identified as *Rhizophagus intraradices*, one of the most widespread AMF. It has been observed in a wide range of natural and anthropogenic ecosystems, from forests and grasslands to orchards and arable fields. VTX0013 also occurs in high-input agricultural ecosystems, suggesting it tolerates high nutrient levels in the soils (Hijri et al., 2006). Indeed, Sylvia and Schenk (1983) showed that P enrichment did not affect sporulation of *Rhizophagus intraradices*. Additionally, at high plant-available P levels, *Rhizophagus intraradices* can even reduce the growth of citrus trees and therefore become an inferior mutualist (Peng et al., 1993). In both observational studies, the degree of nestedness was positively correlated to the available P in the soil. In our field experiment, AMF communities in the inorganic fertilizer treatments were also nested within communities in the slow-release fertilizer treatments. Additionally, the highest available P levels were measured in the inorganic fertilizer treatments. Consequently, chapter three, four and five unambiguously indicate that enrichment of P levels in the soil through fertilization results in a gradual loss of AMF specialists and communities dominated by generalists, such as *Rhizophagus intraradices*, which may be inferior mutualists.

7.3 The effect of organic management on AMF communities

The effects of organic management on AMF diversity were inconclusive. In apple orchards, organic management increased AMF diversity (chapter 3). We found, however, no differences in AMF diversity between conventionally and organically managed vineyards (chapter 5). The main difference between these studies is that the organically managed apple orchards all had low available P levels (< 100 mg P/kg), while available P levels in the soil of organically managed vineyards ranged considerably and did not differ from conventionally managed orchards. In apple

orchards, the difference in AMF diversity between management types may be partly explained by the lower available P in the soils. However, management contributed to AMF diversity even after accounting for available P levels in the soil, indicating that AMF richness of organic orchards were still higher than those of conventionally managed orchards with similar P levels in the soil. This positive effect of organic management may be attributed to the exclusion of chemical biocides and the higher number of weeds present in organically managed orchards (Schreiner & Bethlenfalvay, 1996; Hole *et al.*, 2005).

In vineyards, the positive effect of organic farming on AMF diversity was overruled by the high available P levels in the soil, which did not differ between conventionally and organically managed vineyards (chapter 5). Yet, P is an immobile element and applied P can thus accumulate in the soil as residual P, which can still contribute to the available P in the soil (Sattari *et al.*, 2012). Therefore, a transformation to organic farming will not result in lower available P levels immediately. Although time since conversion to organic farming did not affect AMF diversity, we indeed observed lower available P levels in older organically managed vineyards (Fig. 7.1). In addition, given the build-up of residual P, the starting position of available P levels in the soil, and thus the prehistory of the soil, may also be important to explain current P levels in the soil. Consequently, organic farming on a heavily fertilized agricultural field will take longer to achieve optimal P levels compared to organic farming starting from a grassland with very low available P levels. On the one hand current available P levels in the soil may thus be explained by the time since conversion to organic farming and the prehistory of the soil. On the other hand, organic farming is no guarantee for low available P levels, as organic farming allows organic fertilization, which may still elevate P levels in the soil above optimum levels. Indeed, the soil service of Belgium compared the soil P levels between conventionally and organically managed agricultural fields and pastures and found that despite a slight decrease in soil P levels, circa 60% of the investigated organically managed agricultural fields and pastures had soil P levels above the optimal levels (Tits *et al.*, 2016). This suggests that still high levels of soil P can occur in organically managed fields, which according to our models (chapter 3 and 5), may negatively impact AMF diversity.

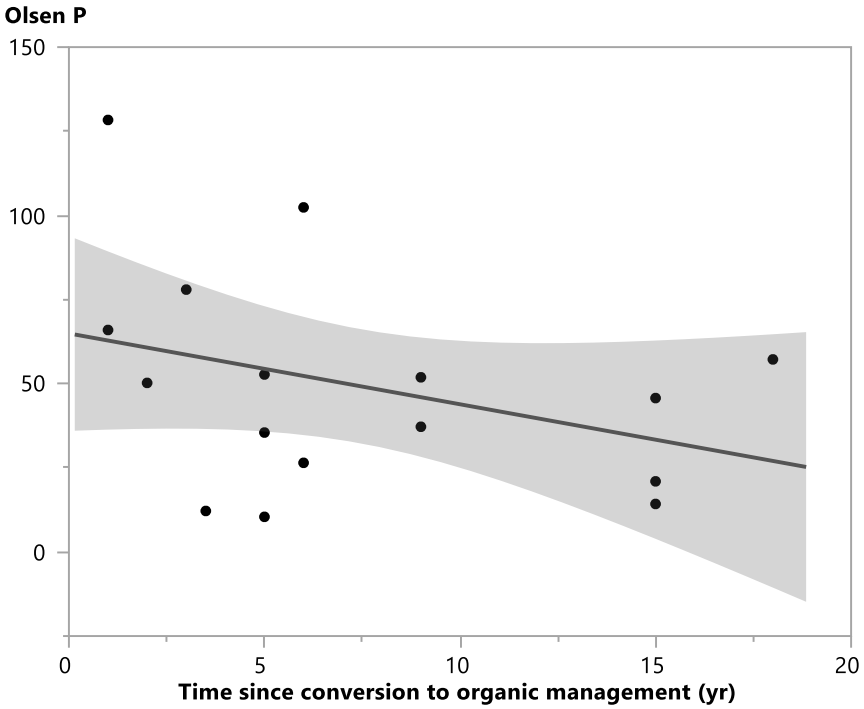


Figure 7.1 The relation between available P levels in 16 vineyard soils and time since conversion to organic management. Olsen P is expressed in mg/kg.

7.4 Conserving AMF diversity in agricultural ecosystems

AMF are among the most ecologically significant organisms on the planet since they act as a living interface between soil and plants, influencing soil fertility, plant nutrition and productivity (van der Heijden *et al.*, 1998; Fitter *et al.*, 2011). They enhance the growth, nutrient uptake and tolerance to abiotic and biotic stresses, compared to non-mycorrhizal plants, and contribute to formation and maintenance of soil structure. These services are provided free to human society and possess a high economic value (Pimentel *et al.*, 1997; Balmford *et al.*, 2002). Yet, the International Union for Conservation of Nature Red List, which is the most valuable guide for estimating endangered species, includes no AMF species. Therefore, AMF are generally excluded in global conservation programs. Given the importance of AMF in plant growth and development, and their role in ecosystem functioning, their conservation is vital in both natural and agricultural ecosystems (Turrini & Giovannetti, 2012).

In this research, we have shown that AMF diversity strongly declines with increasing fertilization and different management practices in agricultural ecosystems (chapter 3-5). Especially, high plant-available P levels in the soil resulted in poor AMF communities dominated by generalists. Therefore, valuable AMF taxa could become extinct before they are even discovered (Turrini & Giovannetti, 2012). In chapter three and five we showed that most AMF diversity occurs when available soil P levels are lower than 70 mg P/kg. In some orchards, however, we measured available P levels up to 200 mg P/kg. According to the model fitted in chapter 3, a reduction of soil P from 200 to 70 mg P/kg will increase AMF diversity by 42%. According to this model, a further reduction of soil P from 200 to 36 mg P/kg, the lowest soil P level measured in an orchard, will increase AMF diversity by

53%. In vineyards, an even stronger relation between soil P levels and AMF richness was found (chapter 5). The highest Olsen P value measured in vineyards was 128 mg P/kg. According to the model fitted in chapter five, a reduction of soil P from 128 mg P/kg to 10 mg P/kg (the lowest soil P level measured in a vineyard), will increase AMF diversity by 63%. In addition, we demonstrated in chapter four that application of slow-release fertilizers, such as struvite and green compost, promoted AMF diversity in comparison to application of inorganic fertilizers that quickly elevated soil P levels. Furthermore, we showed in chapter three that organic management can also have a positive effect on AMF diversity, even when controlling for soil P levels. In chapter five, however, we found that high soil P levels can overrule these positive effects of organic farming. Therefore, we recommend organic management in combination with low to moderate fertilization using slow-release fertilizers to preserve diverse AMF communities in agricultural ecosystems.

7.5 Shortcomings and research perspectives

7.5.1 Method for characterizing AMF communities

In this research, we used next-generation sequencing to characterize AMF communities, which allows accurate and efficient characterization of AMF communities. In chapter two, we evaluated six primer pairs targeting the nuclear rRNA operon for characterization of AMF communities using 454 pyrosequencing. We showed that the primer pair AMV4.5NF-AMDGR, which we used in the subsequent chapters, yielded a high number of high-quality sequences and was highly AMF specific. Yet, our results suggest that this primer pair favored amplification of Glomeraceae sequences at the expense of Ambisporaceae, Claroideoglomeraceae and Paraglomeraceae sequences. Therefore, it is possible that the results obtained in our observational studies (chapter 3 and 5) and field experiment (chapter 4) underestimated the number of AMF taxa belonging to these families. AMF taxa belonging to the Claroideoglomeraceae, however, were always well represented, but low numbers of AMF taxa belonging to the Ambisporaceae and Paraglomeraceae were found in these studies. Nevertheless, it is possible that in orchards and vineyards these taxa do not occur frequently. Indeed, it has been shown repeatedly using various methods to characterize AMF communities that agricultural systems are dominated by AMF belonging to the Glomeraceae (Rosendahl *et al.*, 2009; Verbruggen *et al.*, 2010; Jansa *et al.*, 2014). This is in agreement with our results.

Although the primer pair AMV4.5NF-AMDGR showed to be highly AMF specific (72%) in chapter two, in subsequent chapters AMF specificity varied from 75.4% (chapter 3) and 94.9% (chapter 4) to 28.8% (chapter 5). Therefore, this primer pair can be highly AMF specific, but can sometimes also produce a high number of non-Glomeromycota sequences. When sequencing the roots of *Plantago lanceolata* using the primer pair AMV4.5NF-AMDGR, also large amounts of plant material were detected (data not shown). Therefore caution is necessary when this primer pair is used in other systems. It would thus be interesting to use a primer pair that is even more AMF specific. AML1-AML2 is a good candidate as it showed an AMF specificity of 100% in chapter 2. In addition, it also detects a broad range of AMF taxa, and because of its length (800 bp), it can distinguish between

closely related AMF taxa (Lee *et al.*, 2008). Therefore this primer pair seemed to be a good match for the 454 GS FLX+ system which allows to sequence long reads up to 800 bp. In subsequent trials, however, combining AML1-AML2 with the GS FLS+ system did not yield satisfying results (data not shown). The quality of the reads dropped considerably after 300 bp, leaving a highly variable number of high quality sequences per sample for subsequent analysis. Therefore, to obtain a large number of longer high-quality AMF reads per sample remains a challenge. Primer AML2 can detect a wide range of AMF and is highly AMF specific. Therefore, combining AMV4.5NF with AML2 could result in an interesting new primer combination that amplifies longer fragments (450 bp instead of 300 bp) and is more AMF specific in comparison to AMV4.5NF-AMDGR. This new primer combination, however, has not been used in AMF studies and still needs testing on environmental samples to prove its usefulness.

7.5.2 Sampling design

In chapter three, we sampled five root samples of apple trees and only one soil sample per site. Because of this mismatch between the number of root and soil samples per site, we analyzed our data on the site-level. Therefore, only variation in AMF diversity and community composition could be explained between sites. In chapter five, however, a soil sample was obtained for every root sample. Therefore, it was now possible to analyze the data on the sample-level using mixed model to account for pseudoreplication, and explain variation in AMF diversity and community composition, not only between sites, but also within a site. Consequently, the statistical power increased, which made it possible to detect smaller effects, such the effect of pH on AMF diversity (see 7.2).

7.5.3 How do AMF communities affect crop performance?

In chapter six, we showed that crop biomass increased on average 34.9% in inoculated plants compared to non-inoculated plants, and that a multi-species inoculum decreased the growth response compared to a single species inoculum. Yet, we were unable to measure the effect of changing AMF diversity and community composition as a result of different soil characteristics and management (chapter 3-5) on crop performance of apple trees and grapevines in the field. As crop performance depends both on soil characteristics and management practices, and AMF diversity and community composition, disentangling the effect of the latter is virtually impossible using the sampling design of our observational studies (chapter 3 and 5). Experiments performed in controlled conditions in the greenhouse allow to estimate effects of AMF on crop performance (see chapter 6). Plants in these experiments, however, were grown singly in sieved autoclaved soil mixed with sand. They were kept continuously moist, inoculated with AMF that may not interact with the host in nature and not exposed to other member of natural communities. Although these experiments can provide invaluable insights into the role of AMF in ecosystem functioning (e.g. van der Heijden *et al.*, 1998; Maherali & Klironomos, 2007), they are also performed in quite artificial conditions (Lekberg & Koide, 2014). To what extent complex AMF communities that change with soil conditions and management, contribute to crop performance in the field, however, remains unanswered. A possibility to research the contribution of complex AMF communities in the field, is

to compare a crop with a non-mycorrhizal variant under the same soil conditions and management practices. Barker *et al.* (1998), for example, managed to isolate a mycorrhizal defective mutant of tomato (*Solanum lycopersicum*). This allows to compare the mycorrhizal defective mutant with a mycorrhizal tomato in nutrient rich soils with poor AMF dominated by generalists and nutrient poor soils with rich AMF communities with many specialist species. To our knowledge, no mycorrhizal defective mutants exist for other crops, such as apple trees and grapevines. In addition, tomatoes are generally grown as annuals. Therefore, the mycorrhizal defective mutant allows to assess the effect of complex AMF communities in the field in one growing season. In contrast, apple trees and grapevine are perennial plants often grown up to 20 years. Assessing the contribution of AMF communities in the field to these crops thus requires long term experiments, not feasible in the term of this four year research.

7.6 Main conclusions

Primer pair AMV4.5NF-AMDGR had no or only few mismatches to AMF sequences and was able to consistently describe the AMF communities retrieved from apple orchards. Using this primer pair in both our observational studies, covering apple orchards and vineyards, and our field experiment in an apple orchard, we found that the plant available P in the soil was the most important factor that influenced AMF diversity and community composition. Apart from the available P in the soil, we also established that soil acidity affected AMF diversity. However, this effect was considerably smaller than that of available P and was only revealed after increasing our sample size. Organic farming can have positive effects on AMF diversity as we demonstrated in apple orchards. However, as available P levels in the soil not necessarily differ between conventionally and organically managed agricultural systems (see our vineyard study), the positive effect of organic farming on AMF diversity can be overruled by high available P levels in the soil. To preserve AMF communities in orchards and vineyards, we recommend a combination of organic management with low to moderate fertilization, using slow-release fertilizers.

Our meta-analysis showed that crop biomass increased on average with 34.9% in crop plants that were inoculated with AMF compared to non-inoculated plants, and that a multi-species inoculum decreased the growth response compared to a single species inoculum. The experiments included in our meta-analysis were performed in the greenhouse and thus under stable environmental conditions. To what extent more diverse AMF communities contribute to long term crop performance under variable field conditions remains a promising domain of further study. Given the highly diverse functional diversity of AMF, with some taxa even reported to be parasitic, a focus on dedicated inocula composed of a limited number of beneficial taxa seems appropriate.

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Appendices

Appendix A

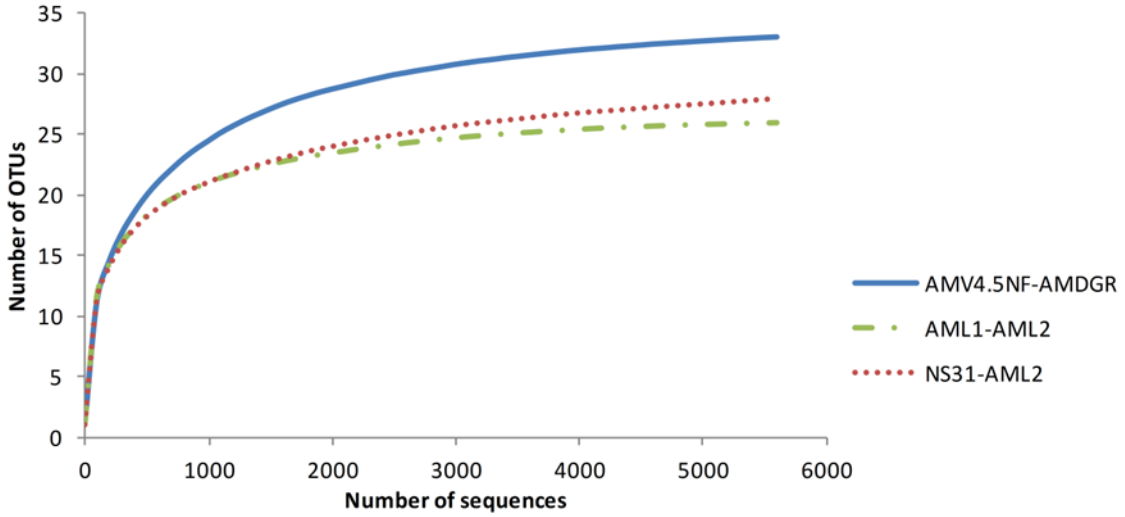


Figure A1 Rarefaction curves generated for the primer pairs AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2.

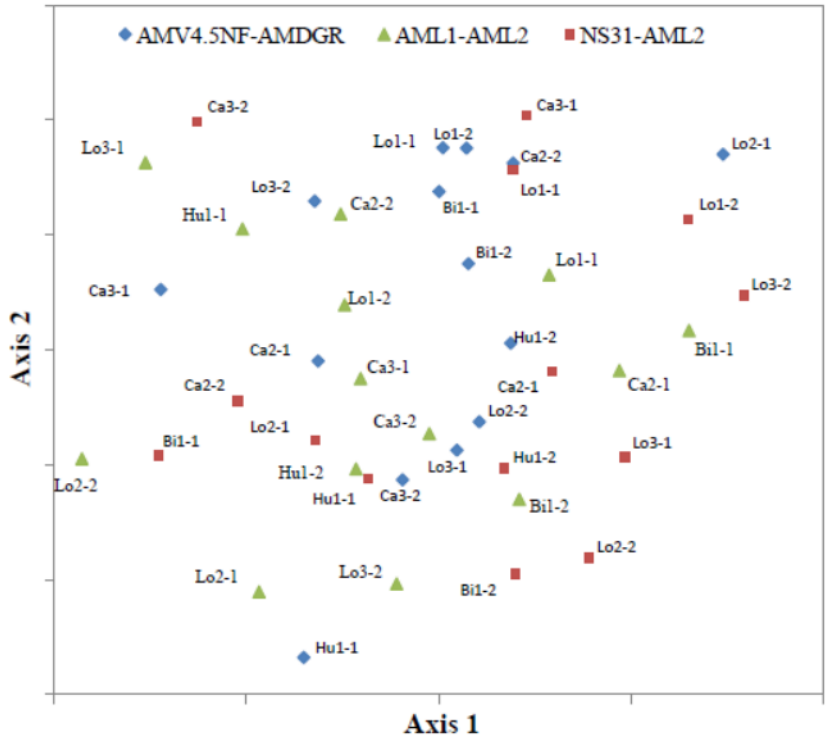


Figure A2 Nonmetric multidimensional scaling ordination of the AMF communities described with the primer pairs AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 based on eight root samples, an 190 bp overlapping SSU rRNA gene fragment and 350 AMF sequences per sample. Final stress value of the ordination was 9.68. Labels show sample ID and replicate number. Samples from the Wi site are not shown to aid visual representation.

Table A1 Pyrosequencing fusion primer component sequences and structure[a]

454 GS-FLX -Lib-L Adaptor Sequences

Application	*Adapter*	*Sequence(5'-3')*
ForwardFusionPrimers	Adaptor A	CCATCTCATCCCTGCGTGTCTCCGACTCAG
ReverseFusionPrimers	Adapter B	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG

Primer Sequences

Combination	*Target*[b]	*Primer*	*Sequence(5'-3')*	*Direction*
AM1-NS31	SSU	AM1	GTTTCCCGTAAGGCGCCGAA	Forward
		NS31	TTGGAGGGCAAGTCTGGTGCC	Reverse
AMV4.5NF-AMDGR	SSU	AMV4.5NF	AAGCTCGTAGTTGAATTTCG	Forward
		AMDGR	CCCAACTATCCCTATTAATCAT	Reverse
AML2-AML1	SSU	AML2	GAACCCAAACACTTTGGTTTCC	Forward
		AML1	ATCAACTTTCGATGGTAGGATAGA	Reverse
AML2-NS31	SSU	AML2	GAACCCAAACACTTTGGTTTCC	Forward
		NS31	TTGGAGGGCAAGTCTGGTGCC	Reverse
FLR3-LSUmBr	LSU	FLR3	TTGAAAGGGAAACGATTGAAGT	Forward
		LSUmBr1	DAACACTCGCATATATGTTAGA	Reverse
		LSUmBr2	AACACTCGCACACATGTTAGA	Reverse
		LSUmBr3	AACACTCGCATACATGTTAGA	Reverse
		LSUmBr4	AAACACTCGCACATATGTTAGA	Reverse
		LSUmBr5	AACACTCGCATATATGCTAGA	Reverse
Glo454-NDL22	LSU	Glo454	TGAAAGGGAAACGATTGAAGT	Forward
		NDL22	TGGTCCGTGTTTCAAGACG	Reverse

Multiplex Identifier Sequences (MID)[c]

1=ACGAGTGCGT	13=CATAGTAGTG	25=TCGTCGCTCG
2=ACGCTCGACA	14=CGAGAGATAC	26=ACATACGCGT
3=AGACGCACTC	15=ATACGACGTA	27=ACGCGAGTAT
4=AGCACTGTAG	16=TCACGTACTA	28=ACTACTATGT
5=ATCAGACACG	17=CGTCTAGTAC	29=ACTGTACAGT
6=ATATCGCGAG	18=TCTACGTAGC	30=AGACTATACT
7=CGTGTCTCTA	19=TGTACTACTC	31=AGCGTCGTCT
8=CTCGCGTGTC	20=ACGACTACAG	32=AGTACGCTAT
9=TAGTATCAGC	21=CGTAGACTAG	33=ATAGAGTACT
10=TCTCTATGCG	22=TACGAGTATG	34=CACGCTACGT
11=TGATACGTCT	23=TACTCTCGTG	35=CAGTAGACGT
12=TACTGAGCTA	24=TAGAGACGAG	36=CGACGTGACT

Fusion Primer Structure

Forward fusion primer:
5'–Adapter A-MID–ForwardPrimer–3'

Reverse fusion primer:
5'–AdapterB–ReversePrimer–3'

a Fusion Primers were designed to meet Roche GS-FLX XLR70 instrument and Lib-L Titanium chemistry requirements (Roche Applied Science, Mannheim, Germany).
b SSU, small subunit rRNA gene; LSU, large subunit rRNA gene.
c Multiplex identifier sequencers were selected from extended MID set TCB No: 005-2009 (Roche Applied Science, Mannheim, Germany)

Table A2 Results of the PrimerProspector analysis

		Sequence (#)	Mismatches (%)						Mismatches (position)			Primer Prospector Score (%)		
Primer	Family	Krüger database	0	1	2	3	4	5+	Outside 3'	3' region (5 bp) except last base	3' terminus	<1	>=1 and <=2	>2
NS31	Acaulosporaceae	40	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NS31	Ambisporaceae	68	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	98.5	1.5	0.0
NS31	Archaeosporaceae	3	66.7	33.3	0.0	0.0	0.0	0.0	33.3	0.0	0.0	100.0	0.0	0.0
NS31	Claroideoglomeraceae	12	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NS31	Diversisporaceae	109	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NS31	Geosiphonoceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NS31	Gigasporaceae	87	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NS31	Glomeraceae	170	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	91.8	8.2	0.0
NS31	Pacisporaceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NS31	Paraglomeraceae	2	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NS31	Total(average of fam)	493	96.7	3.3	0.0	0.0	0.0	0.0	3.3	0.0	0.0	99.0	1.0	0.0
AM1	Acaulosporaceae	40	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AM1	Ambisporaceae	0												
AM1	Archaeosporaceae	0												
AM1	Claroideoglomeraceae	12	0.0	0.0	100.0	0.0	0.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0
AM1	Diversisporaceae	111	47.7	50.5	1.8	0.0	0.0	0.0	52.3	0.0	0.0	100.0	0.0	0.0
AM1	Geosiphonoceae	0												
AM1	Gigasporaceae	105	99.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	61.0	39.0	0.0
AM1	Glomeraceae	170	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AM1	Pacisporaceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AM1	Paraglomeraceae	0												
AM1	Total(average of fam)	439	74.5	8.4	17.0	0.2	0.0	0.0	25.5	16.7	0.0	76.8	23.2	0.0
AMV4.5NF	Acaulosporaceae	41	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMV4.5NF	Ambisporaceae	68	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMV4.5NF	Archaeosporaceae	2	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMV4.5NF	Claroideoglomeraceae	12	0.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0
AMV4.5NF	Diversisporaceae	109	94.5	5.5	0.0	0.0	0.0	0.0	4.6	0.9	0.0	99.1	0.9	0.0
AMV4.5NF	Geosiphonoceae	0												
AMV4.5NF	Gigasporaceae	104	11.5	88.5	0.0	0.0	0.0	0.0	0.0	88.5	0.0	11.5	88.5	0.0
AMV4.5NF	Glomeraceae	170	65.3	34.7	0.0	0.0	0.0	0.0	0.0	34.7	0.0	65.3	34.7	0.0
AMV4.5NF	Pacisporaceae	1	0.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	100.0	0.0	0.0
AMV4.5NF	Paraglomeraceae	0												
AMV4.5NF	Total(average of fam)	507	58.9	41.1	0.0	0.0	0.0	0.0	0.6	40.5	0.0	72.0	28.0	0.0
AMDGR	Acaulosporaceae	41	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Ambisporaceae	52	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Archaeosporaceae	3	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Claroideoglomeraceae	12	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Diversisporaceae	109	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Geosiphonoceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Gigasporaceae	104	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Glomeraceae	170	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Pacisporaceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Paraglomeraceae	2	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AMDGR	Total(average of fam)	495	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML1	Acaulosporaceae	41	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML1	Ambisporaceae	68	83.8	16.2	0.0	0.0	0.0	0.0	0.0	16.2	0.0	83.8	16.2	0.0
AML1	Archaeosporaceae	3	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0
AML1	Claroideoglomeraceae	12	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML1	Diversisporaceae	109	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML1	Geosiphonoceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML1	Gigasporaceae	127	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML1	Glomeraceae	170	99.4	0.6	0.0	0.0	0.0	0.0	0.6	0.0	0.0	100.0	0.0	0.0
AML1	Pacisporaceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML1	Paraglomeraceae	3	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML1	Total(average of fam)	535	88.3	11.7	0.0	0.0	0.0	0.0	0.1	1.6	10.0	88.4	1.6	10.0
AML2	Acaulosporaceae	34	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML2	Ambisporaceae	68	1.5	97.1	1.5	0.0	0.0	0.0	98.5	1.5	0.0	98.5	1.5	0.0
AML2	Archaeosporaceae	2	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML2	Claroideoglomeraceae	12	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML2	Diversisporaceae	109	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML2	Geosiphonoceae	1	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
AML2	Gigasporaceae	104	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML2	Glomeraceae	170	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML2	Pacisporaceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML2	Paraglomeraceae	2	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
AML2	Total(average of fam)	503	80.1	19.7	0.1	0.0	0.0	0.0	19.9	0.1	0.0	99.9	0.1	0.0
FLR3	Acaulosporaceae	125	72.0	28.0	0.0	0.0	0.0	0.0	3.2	24.8	0.0	75.2	24.8	0.0

FLR3	Ambisporaceae	0												
FLR3	Archaeosporaceae	1	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
FLR3	Claroideoglomeraceae	96	97.9	1.0	1.0	0.0	0.0	0.0	1.0	1.0	0.0	99.0	1.0	0.0
FLR3	Diversisporaceae	139	98.6	0.7	0.7	0.0	0.0	0.0	1.4	0.0	0.0	100.0	0.0	0.0
FLR3	Geosiphonoceae	0												
FLR3	Gigasporaceae	181	73.5	26.5	0.0	0.0	0.0	0.0	22.7	3.9	0.0	96.1	3.9	0.0
FLR3	Glomeraceae	94	67.0	33.0	0.0	0.0	0.0	0.0	0.0	33.0	0.0	67.0	33.0	0.0
FLR3	Pacisporaceae	1	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
FLR3	Paraglomeraceae	14	78.6	21.4	0.0	0.0	0.0	0.0	21.4	0.0	0.0	100.0	0.0	0.0
FLR3	Total(average of fam)	651	60.9	38.8	0.2	0.0	0.0	0.0	31.2	7.8	0.0	92.2	7.8	0.0
LSUmBr1	Acaulosporaceae	55	1.8	36.4	61.8	0.0	0.0	0.0	98.2	0.0	0.0	100.0	0.0	0.0
LSUmBr1	Ambisporaceae	9	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr1	Archaeosporaceae	10	0.0	40.0	60.0	0.0	0.0	0.0	100.0	0.0	0.0	40.0	60.0	0.0
LSUmBr1	Claroideoglomeraceae	31	6.5	93.5	0.0	0.0	0.0	0.0	93.5	0.0	0.0	6.5	93.5	0.0
LSUmBr1	Diversisporaceae	30	6.7	93.3	0.0	0.0	0.0	0.0	93.3	0.0	0.0	100.0	0.0	0.0
LSUmBr1	Geosiphonoceae	6	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr1	Gigasporaceae	59	0.0	94.9	5.1	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr1	Glomeraceae	73	95.9	4.1	0.0	0.0	0.0	0.0	2.7	1.4	0.0	98.6	1.4	0.0
LSUmBr1	Pacisporaceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
LSUmBr1	Paraglomeraceae	1	0.0	0.0	100.0	0.0	0.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0
LSUmBr1	Total(average of fam)	275	21.1	46.2	32.7	0.0	0.0	0.0	78.8	10.1	0.0	74.5	25.5	0.0
LSUmBr2	Acaulosporaceae	55	58.2	40.0	1.8	0.0	0.0	0.0	41.8	0.0	0.0	100.0	0.0	0.0
LSUmBr2	Ambisporaceae	13	0.0	7.7	92.3	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr2	Archaeosporaceae	10	0.0	0.0	40.0	60.0	0.0	0.0	100.0	10.0	0.0	40.0	60.0	0.0
LSUmBr2	Claroideoglomeraceae	31	0.0	0.0	6.5	93.5	0.0	0.0	100.0	93.5	0.0	6.5	93.5	0.0
LSUmBr2	Diversisporaceae	30	0.0	93.3	6.7	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr2	Geosiphonoceae	6	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr2	Gigasporaceae	59	5.1	94.9	0.0	0.0	0.0	0.0	94.9	0.0	0.0	100.0	0.0	0.0
LSUmBr2	Glomeraceae	76	0.0	1.3	96.1	2.6	0.0	0.0	100.0	2.6	0.0	96.1	3.9	0.0
LSUmBr2	Pacisporaceae	1	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr2	Paraglomeraceae	1	0.0	0.0	100.0	0.0	0.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0
LSUmBr2	Total(average of fam)	282	6.3	23.7	54.3	15.6	0.0	0.0	93.7	20.6	0.0	74.3	25.7	0.0
LSUmBr3	Acaulosporaceae	56	8.9	58.9	32.1	0.0	0.0	0.0	91.1	0.0	0.0	100.0	0.0	0.0
LSUmBr3	Ambisporaceae	13	0.0	7.7	7.7	84.6	0.0	0.0	100.0	0.0	0.0	15.4	84.6	0.0
LSUmBr3	Archaeosporaceae	10	0.0	0.0	0.0	40.0	60.0	0.0	100.0	10.0	0.0	0.0	90.0	10.0
LSUmBr3	Claroideoglomeraceae	31	0.0	6.5	93.5	0.0	0.0	0.0	100.0	93.5	0.0	6.5	93.5	0.0
LSUmBr3	Diversisporaceae	30	6.7	6.7	86.7	0.0	0.0	0.0	93.3	0.0	0.0	100.0	0.0	0.0
LSUmBr3	Geosiphonoceae	6	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0
LSUmBr3	Gigasporaceae	59	94.9	5.1	0.0	0.0	0.0	0.0	5.1	0.0	0.0	100.0	0.0	0.0
LSUmBr3	Glomeraceae	73	0.0	97.3	2.7	0.0	0.0	0.0	100.0	1.4	0.0	98.6	1.4	0.0
LSUmBr3	Pacisporaceae	1	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr3	Paraglomeraceae	1	0.0	0.0	0.0	100.0	0.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0
LSUmBr3	Total(average of fam)	280	11.1	28.2	22.3	32.5	6.0	0.0	88.9	20.5	0.0	52.0	47.0	1.0
LSUmBr4	Acaulosporaceae	55	30.9	54.5	10.9	3.6	0.0	0.0	69.1	0.0	0.0	96.4	3.6	0.0
LSUmBr4	Ambisporaceae	13	7.7	92.3	0.0	0.0	0.0	0.0	92.3	0.0	0.0	100.0	0.0	0.0
LSUmBr4	Archaeosporaceae	10	0.0	40.0	60.0	0.0	0.0	0.0	100.0	10.0	0.0	90.0	10.0	0.0
LSUmBr4	Claroideoglomeraceae	31	0.0	0.0	64.5	35.5	0.0	0.0	100.0	93.5	0.0	6.5	93.5	0.0
LSUmBr4	Diversisporaceae	30	86.7	6.7	6.7	0.0	0.0	0.0	13.3	0.0	0.0	100.0	0.0	0.0
LSUmBr4	Geosiphonoceae	6	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr4	Gigasporaceae	59	0.0	0.0	6.8	93.2	0.0	0.0	100.0	0.0	0.0	6.8	93.2	0.0
LSUmBr4	Glomeraceae	73	1.4	63.0	35.6	0.0	0.0	0.0	98.6	1.4	0.0	98.6	1.4	0.0
LSUmBr4	Pacisporaceae	1	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr4	Paraglomeraceae	1	0.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0
LSUmBr4	Total(average of fam)	279	12.7	55.7	18.4	13.2	0.0	0.0	77.3	20.5	0.0	69.8	30.2	0.0
LSUmBr5	Acaulosporaceae	55	0.0	1.8	40.0	58.2	0.0	0.0	98.2	100.0	0.0	0.0	100.0	0.0
LSUmBr5	Ambisporaceae	13	0.0	7.7	7.7	84.6	0.0	0.0	92.3	100.0	0.0	0.0	100.0	0.0
LSUmBr5	Archaeosporaceae	10	0.0	0.0	0.0	40.0	60.0	0.0	100.0	100.0	0.0	0.0	40.0	60.0
LSUmBr5	Claroideoglomeraceae	31	93.5	6.5	0.0	0.0	0.0	0.0	0.0	6.5	0.0	93.5	6.5	0.0
LSUmBr5	Diversisporaceae	30	0.0	6.7	93.3	0.0	0.0	0.0	93.3	100.0	0.0	0.0	100.0	0.0
LSUmBr5	Geosiphonoceae	6	0.0	0.0	0.0	100.0	0.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0
LSUmBr5	Gigasporaceae	59	0.0	0.0	94.9	5.1	0.0	0.0	100.0	100.0	0.0	0.0	100.0	0.0
LSUmBr5	Glomeraceae	73	1.4	97.3	1.4	0.0	0.0	0.0	1.4	98.6	0.0	1.4	98.6	0.0
LSUmBr5	Pacisporaceae	1	0.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0
LSUmBr5	Paraglomeraceae	1	0.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
LSUmBr5	Total(average of fam)	279	9.5	32.0	23.7	28.8	6.0	0.0	68.5	80.5	0.0	19.5	74.5	6.0
Glo454	Acaulosporaceae	125	97.6	2.4	0.0	0.0	0.0	0.0	2.4	0.0	0.0	100.0	0.0	0.0
Glo454	Ambisporaceae	29	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0
Glo454	Archaeosporaceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Glo454	Claroideoglomeraceae	96	90.6	8.3	1.0	0.0	0.0	0.0	2.1	0.0	7.3	92.7	0.0	7.3
Glo454	Diversisporaceae	139	52.5	47.5	0.0	0.0	0.0	0.0	4.3	22.3	20.9	56.8	22.3	20.9
Glo454	Geosiphonoceae	6	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0
Glo454	Gigasporaceae	182	84.1	15.4	0.5	0.0	0.0	0.0	7.1	7.7	1.1	91.2	7.1	1.6
Glo454	Glomeraceae	223	33.2	66.8	0.0	0.0	0.0	0.0	14.3	11.2	41.3	47.5	11.2	41.3
Glo454	Pacisporaceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Glo454	Paraglomeraceae	14	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0

Glo454	Total(average of fam)	816	65.8	34.0	0.2	0.0	0.0	0.0	3.0	4.1	27.1	68.8	4.1	27.1
NDL22	Acaulosporaceae	82	97.6	1.2	1.2	0.0	0.0	0.0	1.2	1.2	0.0	98.8	1.2	0.0
NDL22	Ambisporaceae	26	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NDL22	Archaeosporaceae	10	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NDL22	Claroideoglomeraceae	43	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NDL22	Diversisporaceae	94	98.9	1.1	0.0	0.0	0.0	0.0	0.0	1.1	0.0	98.9	1.1	0.0
NDL22	Geosiphonoceae	6	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NDL22	Gigasporaceae	138	97.1	0.0	2.9	0.0	0.0	0.0	2.9	0.0	2.9	97.1	0.0	2.9
NDL22	Glomeraceae	225	97.8	1.8	0.4	0.0	0.0	0.0	2.2	0.0	0.0	100.0	0.0	0.0
NDL22	Pacisporaceae	1	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NDL22	Paraglomeraceae	10	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
NDL22	Total(average of fam)	635	99.1	0.4	0.5	0.0	0.0	0.0	0.6	0.2	0.3	99.5	0.2	0.3

Table A3 Amplicons obtained with the six different primer pairs used in this study

Site ID	Sample ID	NS31 -AM1	AMV4.5NF -AMDGR	Glo454 -NDL22	NS31 -AML2	FLR3 -LSUmBr	AML1 -AML2
Bi	Bi1, Bi2, Bi3	+,+,+	+,+,+	+,+,+	+,+,+	+,+,+	+,+,+
Ca	Ca1,Ca2, Ca3	+,+,+	+,+,+	+,+,+	+,+,+	+,+,+	+,+,+
Ha	Ha1,Ha2, Ha3	+,+,+	+,+,+	+,+,+	+,-,-	+,+,+	+,-,-
Hu	Hu1, Hu2, Hu3	+,+,+	+,+,+	+,+,+	+,+,-	+,+,+	+,+,-
Lo	Lo1, Lo2, Lo3	+,+,+	+,+,+	+,+,+	+,+,+	+,+,+	+,+,+
Wi	Wi1, Wi2, Wi3	+,+,+	+,+,+	+,+,+	+,+,+	+,+,+	+,+,+

+, PCR amplicon obtained;
-, no amplicon obtained (as assessed by agarose gel electrophoresis).

Table A4 Number of AMF sequences obtained per sample

	AMV4.5NF-AMDGR				AML1-AML2				NS31-AML2			
Sample	Rep1	Rep2	Mean	Total	Rep1	Rep2	Mean	Total	Rep1	Rep2	Mean	Total
Bi1	798	864	831	1662	6165	538	3352	6703	1701	105	903	1806
Bi2	410	1233	822	1643	4	1494	749	1498	9	400	205	409
Bi3	2270	1450	1860	3720	25	540	283	565	25	4355	2190	4380
Ca1	1639	1999	1819	3638	2409	4436	3423	6845	157	2815	1486	2972
Ca2	1269	1259	1264	2528	5180	3673	4427	8853	931	5883	3407	6814
Ca3	1267	1151	1209	2418	1645	x	1645	1645	1917	11	964	1928
Ha1	1277	1621	1449	2898	x	x	x	x	x	x	x	x
Ha2	1247	1398	1323	2645	x	x	x	x	x	x	x	x
Ha3	1011	1733	1372	2744	15	345	180	360	1	4899	2450	4900
Hu1	830	586	708	1416	5203	427	2815	5630	2452	462	1457	2914
Hu2	580	559	570	1139	2369	x	2369	2369	40	5	23	45
Hu3	588	649	619	1237	x	x	x	x	x	x	x	x
Lo1	1361	2306	1834	3667	802	5590	3196	6392	5097	959	3028	6056
Lo2	1516	2956	2236	4472	6002	10724	8363	16726	1237	3056	2147	4293
Lo3	20590	4255	12423	24845	528	11957	6243	12485	6605	806	3706	7411
Wi1	2336	4468	3402	6804	1075	89	582	1164	2227	475	1351	2702
Wi2	714	x	714	714	5	x	5	5	71	88	80	159
Wi3	933	540	737	1473	1638	491	1065	2129	744	459	602	1203
Total	40636	29027		69663	33065	40304		73369	23214	24778		47992

x, No AMF sequences obtained.

Table A5 List of operational taxonomic units (OTUs), identified at a 3% sequence dissimilarity cut-off discovered by the primer pairs AMV4.5NF-AMDGR, AML1-AML2 and NS31-AML2 based on 8 samples (two replicates), an 190 bp overlapping SSU rRNA gene fragment and 350 AMF sequences per sample. The taxonomic affiliations were obtained by BLAST analysis against Genbank and the Krüger *et al*.. (2012) database.

	OTU obtained with				Taxonomic affiliation	Genbank					Krüger Database				
OTU No.	AMV4.5NF-AMDGR	AML1-AML2	NS31-AML2	No. of sequences	Family	Closest match	Accession No.	Bitscore	E-value	Sequence identity	Closest match	Sequence ID	Bitscore	E-value	Sequence identity
OTU_1	x	x	x	4604	Claroideoglomeraceae	Glomus sp.	AB569109.1	319	8.80E-84	185/190	Claroideoglomus	Fi5cons#04	261	3E-71	183/190
OTU_2	x	x	x	3764	Glomeraceae	Rhizophagus intraradices	JX049527.1	352	8.67E-94	190/190	Rhizophagus	FJ009618	351	2E-98	190/190
OTU_6	x	x	x	2084	Gigasporaceae	Scutellospora calospora	FJ009672.1	346	4.03E-92	190/190	Scutellospora	AF074342	329	9E-92	187/190
OTU_209		x	x	1699	Claroideoglomeraceae	Glomus sp.	AB569109.1	322	6.80E-85	186/190	Claroideoglomus	Fi5cons#04	279	9E-77	182/190
OTU_8	x	x	x	1065	Paraglomeraceae	Paraglomus	FN388939.1	351	8.67E-94	190/190	Paraglomus	Fi3cons#09	329	9E-92	186/190
OTU_81	x	x	x	878	Glomeraceae	Glomus sp.	JQ811204.1	339	6.75E-90	188/190	Rhizophagus	FJ009618	333	7E-93	187/190
OTU_89	x	x	x	637	Glomeraceae	Glomus irregulare	FJ009612.1	346	4.03E-92	190/190	Rhizophagus	FJ009612	346	9E-97	190/190
OTU_240	x	x	x	614	Glomeraceae	Glomus irregulare	FJ009612.1	335	8.73E-89	187/190	Rhizophagus	FJ009612	335	2E-93	187/190
OTU_12	x	x	x	334	Claroideoglomeraceae	Glomus sp.	AB569109.1	302	8.86E-79	184/190	Claroideoglomus	Fi5cons#04	246	1E-66	181/190
OTU_231	x	x	x	270	Glomeraceae	Rhizophagus intraradices	JX051853.1	341	1.88E-90	189/190	Rhizophagus	FJ009617	340	4E-95	189/190
OTU_212	x	x	x	142	Claroideoglomeraceae	Glomus sp.	AB569109.1	293	5.33E-76	187/190	Claroideoglomus	Fi5cons#04	235	2E-63	181/190
OTU_137	x	x	x	119	Glomeraceae	Glomus irregulare	FJ009612.1	343	5.22E-91	187/190	Rhizophagus	FJ009612	342	1E-95	187/190
OTU_43	x	x	x	103	Claroideoglomeraceae	Glomus sp.	AF481689.1	337	2.43E-89	189/190	Claroideoglomus	FM876807_R	320	6E-89	186/190
OTU_45	x	x	x	81	Glomeraceae	Glomus clarum	AJ505619.1	352	8.67E-94	190/190	Funneliformis	FN547499_R	351	2E-98	190/190
OTU_90	x	x	x	80	Claroideoglomeraceae	Glomus sp.	JQ237137.1	324	1.89E-85	186/190	Claroideoglomus	FM876807_R	255	2E-69	180/190
OTU_141		x	x	78	Claroideoglomeraceae	Glomus sp.	AB569109.1	319	8.80E-84	186/190	Claroideoglomus	Fi5cons#04	274	4E-75	179/190
OTU_40	x	x	x	71	Diversisporaceae	Diversispora epigaea	FR686936.1	352	8.67E-94	190/190	Diversispora	FR686941_R	346	9E-97	189/190
OTU_27	x	x	x	30	Glomeraceae	Glomus sp.	EU518491.1	348	1.12E-92	188/190	Rhizophagus	GQ205078_R	267	7E-73	185/190
OTU_132	x	x	x	26	Claroideoglomeraceae	Glomus sp.	AB569109.1	308	1.90E-80	184/190	Claroideoglomus	Fi5cons#04	250	7E-68	180/190
OTU_83	x	x	x	21	Claroideoglomeraceae	Glomus sp.	AB076344.1	330	4.06E-87	186/190	Claroideoglomus	FM876807_R	279	9E-77	182/190
OTU_224	x	x	x	13	Claroideoglomeraceae	Glomus sp.	AB569109.1	286	8.92E-74	180/190	Claroideoglomus	Fi5cons#04	241	4E-65	182/190
OTU_22	x		x	12	Glomeraceae	Glomus sp.	AF074370.1	346	4.03E-92	190/190	Rhizophagus	GQ205078_R	252	2E-68	183/190
OTU_134		x	x	11	Ambisporaceae	Archaeosporales sp.	AB490495.1	334	3.14E-88	187/190	Ambispora	Fi3cons#02	254	2E-54	182/190
OTU_232	x			9	Glomeraceae	Glomus sp.	EU518491.1	324	1.89E-85	185/190	Rhizophagus	GQ205078_R	278	3E-76	180/190
OTU_221	x	x		9	Glomeraceae	Glomus sp.	AJ715997.1	346	4.03E-92	189/190	Rhizophagus	Fi1cons#32	335	2E-93	187/190
OTU_214	x		x	7	Claroideoglomeraceae	Glomus sp.	AB569109.1	293	5.33E-76	184/190	Claroideoglomus	Fi5cons#04	237	6E-64	181/190
OTU_181	x			6	Glomeraceae	Glomus fasciculatum	AF231760.1	332	1.13E-87	186/190	Rhizophagus	FJ009612	329	9E-92	186/190
OTU_11	x			5	Glomeraceae	Glomus sp.	AY512367.1	352	8.67E-94	190/190	Rhizophagus	Fi1cons#32	302	2E-83	181/190
OTU_24	x		x	5	Glomeraceae	Glomus macrocarpum	FR772325.1	352	8.67E-94	190/190	Glomus	FR750371_R	344	3E-96	189/190
OTU_254	x	x	x	5	Glomeraceae	Glomus sp.	FN429112.1	324	1.89E-85	186/190	Rhizophagus	FJ009618	318	2E-88	185/190
OTU_10	x		x	4	Claroideoglomeraceae	Glomus sp.	AF074371.1	343	5.22E-91	188/190	Claroideoglomus	FM876807_R	318	2E-88	184/190
OTU_197	x			3	Glomeraceae	Glomus sp.	AJ418880.1	341	1.88E-90	189/190	Sclerocystis	Fi1cons#33	255	2E-69	179/190
OTU_252	x			3	Claroideoglomeraceae	Glomus sp.	AB569109.1	282	1.15E-72	180/190	Claroideoglomus	Fi5cons#04	235	2E-63	182/190
OTU_146	x		x	2	Glomeraceae	Glomus sp.	AF074370.1	341	1.88E-90	189/190	Rhizophagus	GQ205078_R	257	4E-70	176/190
OTU_68		x		2	Diversisporaceae	Glomeromycota clone	GU238365.1	352	8.67E-94	190/190	Diversispora	FN547665_R	340	4E-95	188/190
OTU_123		x		2	Glomeraceae	Glomus sp.	AF481688.1	297	4.12E-77	181/190	Rhizophagus	FJ009618	289	2E-79	179/190
OTU_135	x			1	Glomeraceae	Rhizophagus intraradices	JX049527.1	330	4.06E-87	187/190	Rhizophagus	FJ009618	329	9E-92	187/190
OTU_215	x			1	Claroideoglomeraceae	Glomus sp.	AB569109.1	289	6.90E-75	183/190	Claroideoglomus	Fi5cons#04	228	3E-61	173/190

Appendix B

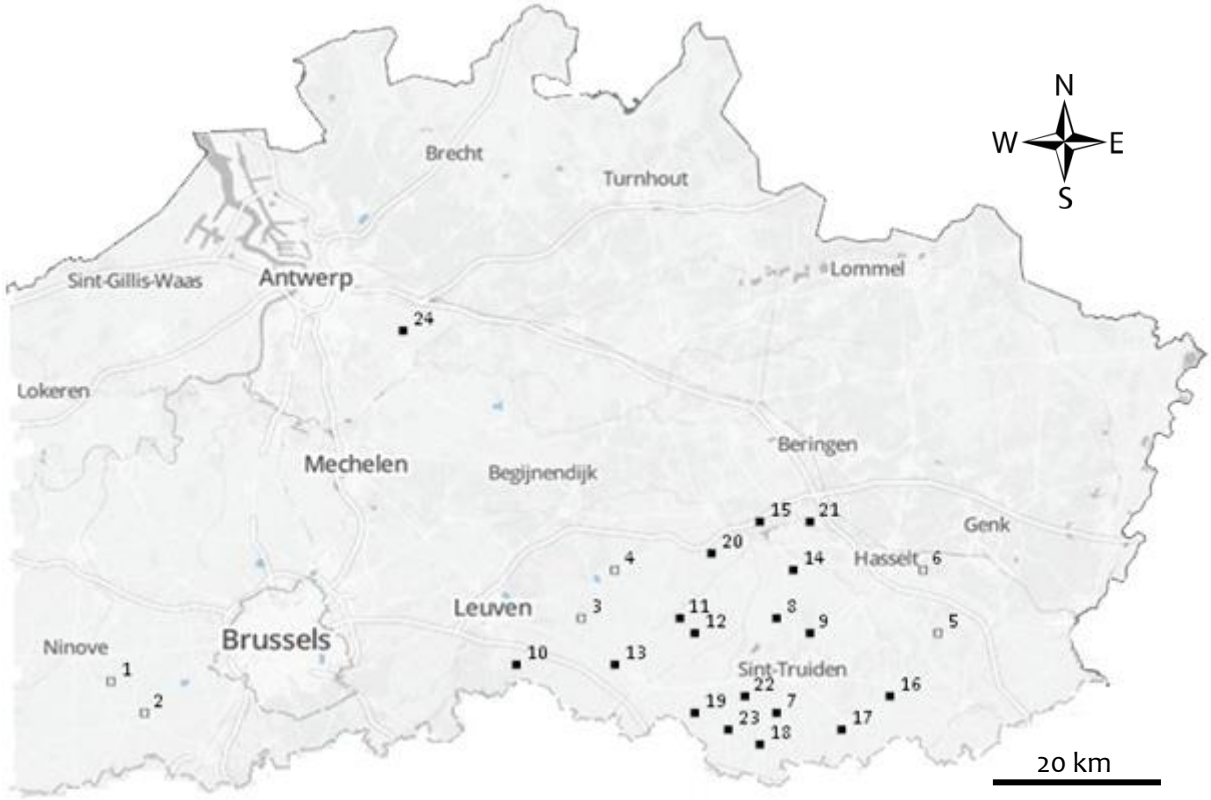


Figure B1 Map of the central and eastern part of Flanders (Belgium) showing the distribution of the 24 sampled apple orchards. White squares: organic, Black squares: conventional.

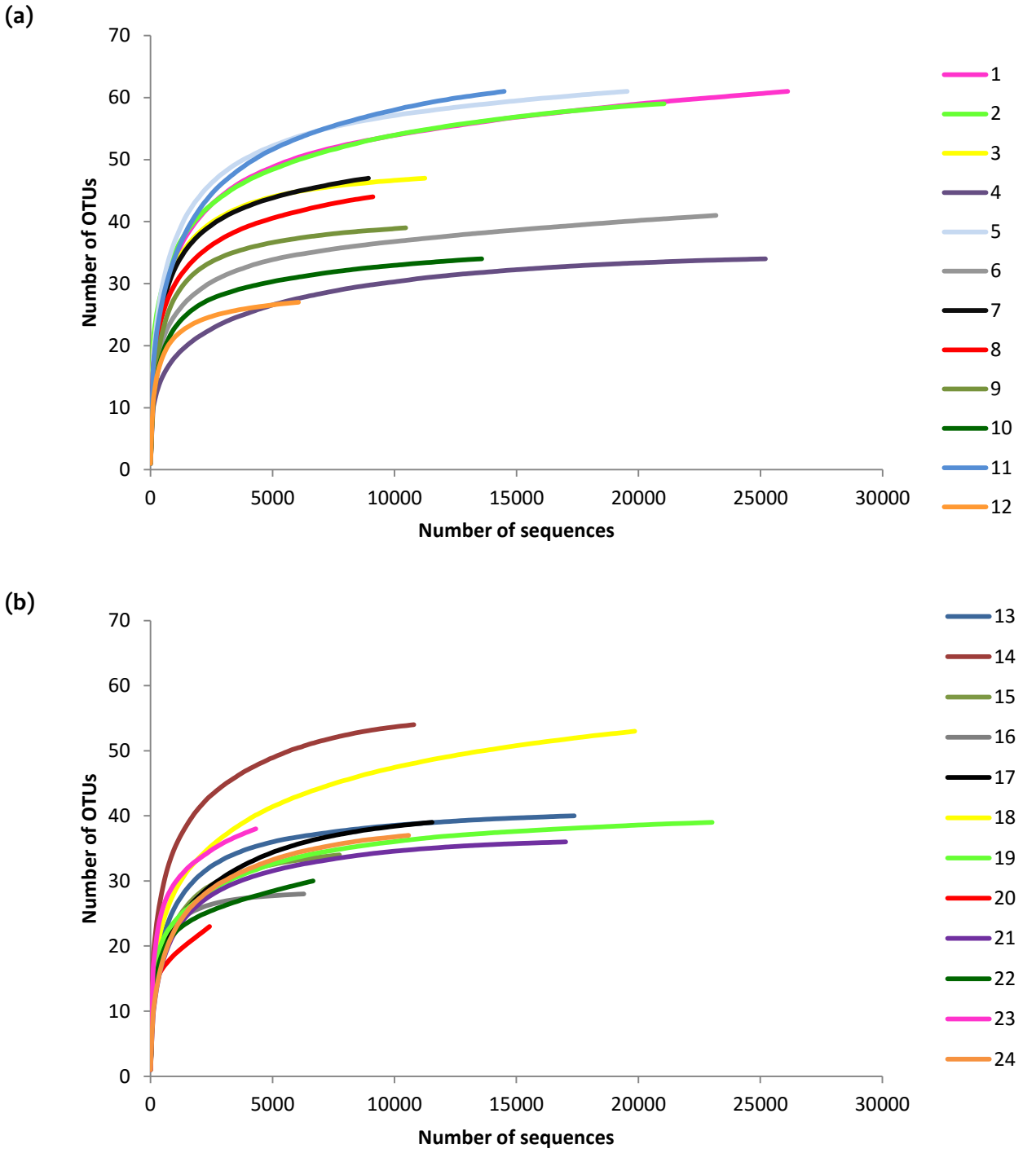


Figure B2 Rarefaction curves of arbuscular mycorrhizal fungal operational taxonomic units for all 24 orchards. For the sake of graphical representation, the curves are shown in two separate graphs, (a) and (b). Orchards are shown in different colors.

Table B1 Coordinates, soil properties, the number of samples remaining after rarefying and AMF diversity measurements of each apple orchard sampled in chapter 3.

Orchard ID	No. of samples after rarefying*	Latitude	Longitude	Soil texture	Management	pH	Olson P (mg/kg)	Soil N (mg/kg)	Organic carbon (%)	AMF richness (S.E)*	Exp(Shannon) (S.E.)*
1	5	50.77	4.08	Loam	Organic	6.2	52.2	4.66	1.17	27.40 (3.29)	5.92 (0.57)
2	5	50.78	4.08	Sandy Loam	Organic	6.5	48.0	5.36	1.31	22.16 (1.56)	6.95 (1.11)
3	3	50.85	4.94	Loam	Organic	7.1	70.6	36.36	1.34	22.58 (3.95)	5.70 (0.91)
4	5	50.88	4.96	Loam	Organic	6.3	66.2	11.89	1.04	18.44 (0.65)	4.78 (0.22)
5	5	50.84	5.43	Loam	Organic	5.1	62.0	2.56	1.17	24.67 (1.21)	5.30 (0.76)
6	5	50.92	5.41	Loam	Organic	4.9	89.0	2.80	1.28	18.75 (0.74)	5.90 (0.41)
7	4	50.77	5.15	Loam	Conventional	6.6	36.6	9.56	1.00	22.76 (3.08)	5.18 (0.88)
8	4	50.86	5.16	Loam	Conventional	6.8	54.4	10.26	1.12	21.87 (1.54)	4.88 (1.05)
9	5	50.86	5.29	Loam	Conventional	6.9	75.0	9.56	1.27	18.96 (3.31)	4.04 (1.08)
10	5	50.82	4.76	Loam	Conventional	6.6	144.6	13.99	1.07	16.44 (0.94)	3.82 (0.51)
11	3	50.88	5.02	Loam	Conventional	7.5	66.8	64.34	1.32	28.16 (5.09)	6.18 (2.08)
12	2	50.86	5.04	Loam	Conventional	7.1	91.6	47.55	1.54	15.94 (1.69)	3.53 (1.31)
13	5	50.82	4.92	Loam	Conventional	7.1	66.8	52.21	1.03	17.89 (1.91)	3.89 (0.73)
14	3	50.92	5.20	Loam	Conventional	6.6	76.6	10.72	1.50	23.86 (5.13)	5.81 (2.66)
15	2	50.95	5.14	Sandy Loam	Conventional	5.9	71.8	20.05	0.97	19.29 (0.26)	5.01 (0.59)
16	2	50.76	5.36	Loam	Conventional	5.8	59.8	26.57	1.21	16.45 (0.45)	6.74 (1.02)
17	4	50.74	5.29	Loam	Conventional	6.1	99.4	11.66	1.30	16.48 (1.25)	4.80 (1.27)
18	5	50.73	5.14	Loam	Conventional	6.9	72.0	9.79	1.08	19.81 (1.76)	4.21 (0.95)
19	4	50.76	5.05	Loam	Conventional	4.9	219.0	41.72	0.84	14.39 (0.97)	3.24 (0.16)
20	1	50.93	5.09	Loam	Conventional	6.7	136.4	94.64	0.97	16.31 (0.00)	4.23 (0.00)
21	4	50.96	5.20	Loam	Conventional	6.5	75.2	10.26	1.13	19.53 (0.93)	2.47 (0.13)
22	3	50.77	5.08	Loam	Conventional	5.1	97.0	19.58	1.42	18.84 (1.27)	4.75 (1.28)
23	3	50.74	5.09	Loam	Conventional	7.8	115.0	13.75	0.88	15.14 (0.72)	5.52 (0.83)
24	3	51.17	4.60	Sandy Loam	Conventional	5.6	203.2	13.52	0.96	18.02 (2.91)	2.93 (0.91)

*After rarefying to 1371 sequences per sample

Table B2 List of the 110 operational taxonomic units (OTUs), identified at a 3% sequence dissimilarity cut-off discovered in chapter 3. The taxonomic affiliations were obtained by BLAST analysis against the MaarjAM database.

OTU ID	Number of reads (unrarefied dataset)	Number of reads. (rarefied dataset)	Genbank accession No.	Closest match Maarjam ID	Family	Genus	MaarjAM Virtual taxa ID	MaarjAM Bitscore	MaarjAM e-value	Identity (bp)	Identity (%)
OTU_1	88802	31052	KJ921497	HE659733	Glomeraceae	Glomus	VTX00113	438	e-123	224/225	99.60%
OTU_2	44290	19609	KJ921518	HE659731	Claroideoglomeraceae	Claroideoglomus	VTX00057	438	e-123	224/225	99.60%
OTU_3	30905	12514	KJ921538	FR848078	Claroideoglomeraceae	Claroideoglomus	VTX00225	432	e-122	225/225	100.00%
OTU_364	29840	11406	KJ921556	HF567017	Glomeraceae	Glomus	VTX00115	414	e-116	221/225	98.20%
OTU_473	22298	8520	KJ921579	HF566978	Glomeraceae	Glomus	VTX00113	414	e-116	221/225	98.20%
OTU_352	8517	2962	KP064038	HF566991	Glomeraceae	Glomus	VTX00114	406	e-114	221/225	98.20%
OTU_6	7810	2236	KJ921593	HF567226	Glomeraceae	Glomus	VTX00212	446	e-126	225/225	100.00%
OTU_24	7748	2488	KJ921525	DQ497054	Glomeraceae	Glomus	VTX00219	432	e-122	225/225	100.00%
OTU_8	6961	3126	KJ921598	GU353747	Claroideoglomeraceae	Claroideoglomus	VTX00225	377	e-105	218/225	96.90%
OTU_10	5988	2427	KJ921498	HF566628	Glomeraceae	Glomus	VTX00163	432	e-122	225/225	100.00%
OTU_22	5687	3434	KJ921521	GU353736	Claroideoglomeraceae	Claroideoglomus	VTX00056	424	e-119	224/225	99.60%
OTU_61	5066	1745	KJ921594	HQ323487	Claroideoglomeraceae	Claroideoglomus	VTX00225	432	e-122	225/225	100.00%
OTU_15	4392	1469	KJ921510	FR848023	Glomeraceae	Glomus	VTX00160	432	e-122	225/225	100.00%
OTU_11	3629	1101	KJ921501	HE799069	Glomeraceae	Glomus	VTX00214	432	e-122	225/225	100.00%
OTU_29	3483	1303	KJ921535	AJ309465	Glomeraceae	Glomus	VTX00143	432	e-122	225/225	100.00%
OTU_453	3116	1228	KP064039	AM849304	Glomeraceae	Glomus	VTX00186	422	e-119	222/225	98.60%
OTU_21	2786	877	KJ921519	EU340323	Claroideoglomeraceae	Claroideoglomus	VTX00193	416	e-117	223/225	99.10%
OTU_33	2575	1964	KJ921548	HE659940	Paraglomeraceae	Paraglomus	VTX00281	446	e-126	225/225	100.00%
OTU_261	2547	880	KJ921528	HM215868	Glomeraceae	Glomus	VTX00214	426	e-120	222/225	98.70%
OTU_437	2409	773	KJ921570	JN090188	Glomeraceae	Glomus	VTX00143	404	e-113	222/225	98.70%
OTU_301	2374	758	KJ921540	HF566988	Glomeraceae	Glomus	VTX00114	406	e-114	220/225	97.80%
OTU_327	2243	872	KJ921547	HE798904	Glomeraceae	Glomus	VTX00115	430	e-121	220/225	97.80%
OTU_38	1839	684	KJ921558	HE798948	Glomeraceae	Glomus	VTX00143	432	e-122	225/225	100.00%
OTU_381	1700	624	KJ921560	DQ164812	Glomeraceae	Glomus	VTX00163	414	e-116	221/225	98.20%
OTU_240	1489	680	KJ921526	HE798863	Glomeraceae	Glomus	VTX00113	404	e-113	222/225	98.70%
OTU_331	1391	387	KJ921550	HE799083	Glomeraceae	Glomus	VTX00219	406	e-114	220/225	97.80%
OTU_388	1384	557	KJ921563	HF566978	Glomeraceae	Glomus	VTX00113	422	e-119	222/225	98.70%
OTU_422	1343	444	KJ921568	EU340323	Claroideoglomeraceae	Claroideoglomus	VTX00193	387	e-108	216/225	96.00%
OTU_470	1327	432	KJ921576	FJ867636	Glomeraceae	Glomus	VTX00113	400	e-112	221/225	98.20%
OTU_478	1135	482	KJ921581	HF567017	Glomeraceae	Glomus	VTX00115	408	e-115	221/225	98.20%
OTU_423	1027	503	KJ921569	FN556647	Glomeraceae	Glomus	VTX00163	404	e-113	220/225	97.80%
OTU_119	954	451	KJ921506	HM215678	Glomeraceae	Glomus	VTX00065	424	e-119	224/225	99.60%
OTU_48	856	408	KJ921582	HE798763	Gigasporaceae	Scutellospora	VTX00049	432	e-122	225/225	100.00%
OTU_47	831	288	KJ921575	HE659720	Glomeraceae	Glomus	VTX00199	446	e-126	225/225	100.00%
OTU_356	711	237	KJ921553	JN090188	Glomeraceae	Glomus	VTX00143	392	e-110	220/225	97.80%
OTU_270	619	320	KJ921530	FM876883	Glomeraceae	Glomus	VTX00342	408	e-115	219/225	97.30%
OTU_117	585	179	KJ921505	FJ867636	Glomeraceae	Glomus	VTX00113	396	e-111	221/225	98.20%
OTU_57	573	179	KJ921592	JN009269	Glomeraceae	Glomus	VTX00125	432	e-122	225/225	100.00%
OTU_63	564	158	KJ921595	HE799164	Glomeraceae	Glomus	VTX00072	438	e-123	224/225	99.60%
OTU_472	513	264	KJ921578	FJ875130	Glomeraceae	Glomus	VTX00163	394	e-110	222/225	98.70%
OTU_89	475	115	KJ921600	FJ194511	Glomeraceae	Glomus	VTX00309	432	e-122	225/225	100.00%
OTU_359	465	184	KJ921555	HF566980	Glomeraceae	Glomus	VTX00113	371	e-103	220/225	97.80%
OTU_485	438	141	KJ921585	EF393582	Glomeraceae	Glomus	VTX00113	406	e-114	220/225	97.80%
OTU_494	419	167	KJ921587	HM215868	Glomeraceae	Glomus	VTX00214	400	e-112	221/225	98.20%
OTU_55	419	203	KJ921591	FR751300	Glomeraceae	Glomus	VTX00067	432	e-122	225/225	100.00%
OTU_70	414	248	KJ921597	GU353928	Glomeraceae	Glomus	VTX00151	432	e-122	225/225	100.00%
OTU_116	403	113	KJ921504	FJ194512	Glomeraceae	Glomus	VTX00309	424	e-119	224/225	99.60%
OTU_108	363	159	KJ921499	FR686936	Diversisporaceae	Diversispora	VTX00061	432	e-122	225/225	100.00%
OTU_335	354	141	KJ921551	HE615072	Claroideoglomeraceae	Claroideoglomus	VTX00193	422	e-119	223/225	99.10%
OTU_65	291	97	KJ921596	HE799033	Glomeraceae	Glomus	VTX00186	416	e-117	224/225	99.60%
OTU_133	268	177	KJ921508	FN869794	Claroideoglomeraceae	Claroideoglomus	VTX00340	432	e-122	225/225	100.00%
OTU_476	263	110	KJ921580	HE659733	Glomeraceae	Glomus	VTX00113	414	e-116	212/225	94.20%
OTU_480	258	128	KJ921583	HF567017	Glomeraceae	Glomus	VTX00115	414	e-116	221/225	98.20%
OTU_350	250	91	KJ921552	FN556647	Glomeraceae	Glomus	VTX00163	406	e-114	215/225	95.60%
OTU_400	229	82	KJ921564	HF567017	Glomeraceae	Glomus	VTX00115	414	e-116	221/225	98.20%
OTU_263	207	84	KJ921529	HE659731	Claroideoglomeraceae	Claroideoglomus	VTX00057	416	e-117	216/225	96.00%
OTU_170	203	107	KJ921512	GU059538	Glomeraceae	Glomus	VTX00222	446	e-126	225/225	100.00%
OTU_416	203	50	KJ921566	HF567017	Glomeraceae	Glomus	VTX00115	424	e-119	220/225	97.80%
OTU_111	174	68	KJ921502	GU353726	Gigasporaceae	Scutellospora	VTX00052	446	e-126	225/225	100.00%
OTU_109	168	73	KJ921500	HE659885	Glomeraceae	Glomus	VTX00064	438	e-123	224/225	99.60%
OTU_114	162	48	KJ921503	AB076344	Claroideoglomeraceae	Claroideoglomus	VTX00278	432	e-122	225/225	100.00%
OTU_383	157	63	KJ921561	HQ656918	Glomeraceae	Glomus	VTX00143	400	e-112	221/225	98.20%
OTU_373	148	51	KJ921557	JN090188	Glomeraceae	Glomus	VTX00143	408	e-115	219/225	97.30%
OTU_98	135	47	KJ921601	GU353746	Glomeraceae	Glomus	VTX00222	432	e-122	225/225	100.00%
OTU_220	123	40	KJ921522	HF567267	Glomeraceae	Glomus	VTX00222	422	e-119	222/225	98.70%
OTU_309	113	59	KJ921543	FJ875130	Glomeraceae	Glomus	VTX00163	412	e-116	215/225	95.60%
OTU_131	108	30	KJ921507	FJ831571	Glomeraceae	Glomus	VTX00155	432	e-122	225/225	100.00%
OTU_140	104	44	KJ921509	AB193051	Claroideoglomeraceae	Claroideoglomus	VTX00279	373	e-104	213/225	94.70%
OTU_164	76	44	KJ921511	FR848069	Glomeraceae	Glomus	VTX00172	430	e-121	224/225	99.60%
OTU_173	75	27	KJ921513	EU340314	Glomeraceae	Glomus	VTX00064	438	e-123	224/225	99.60%
OTU_313	71	19	KJ921544	GU353476	Glomeraceae	Glomus	VTX00309	398	e-112	211/225	93.80%

OTU_330	66	20	KJ921549	JN090188	Glomeraceae	Glomus	VTX00143	394	e-110	222/225	98.70%
OTU_190	63	16	KJ921515	JN252444	Gigasporaceae	Scutellospora	VTX0041	424	e-119	224/225	99.60%
OTU_199	45	35	KJ921517	AB195628	Claroideoglomeraceae	Claroideoglomus	VTX00278	379	e-106	221/225	98.20%
OTU_88	42	10	KJ921599	HE798986	Glomeraceae	Glomus	VTX00163	432	e-122	225/225	100.00%
OTU_185	40	18	KJ921514	GU598368	Glomeraceae	Glomus	VTX00130	438	e-123	224/225	99.60%
OTU_244	40	38	KJ921527	AF074346	Acaulosporaceae	Acaulospora	VTX00030	424	e-119	224/225	99.60%
OTU_505	36	13	KJ921590	EF041097	Claroideoglomeraceae	Claroideoglomus	VTX00225	389	e-109	217/225	96.40%
OTU_302	35	18	KJ921541	AY499494	Glomeraceae	Glomus	VTX00159	402	e-113	223/225	99.10%
OTU_498	32	12	KJ921588	FN556647	Glomeraceae	Glomus	VTX00163	387	e-108	221/225	98.20%
OTU_192	26	2	KJ921516	AJ301856	Claroideoglomeraceae	Claroideoglomus	VTX00193	408	e-115	222/225	98.70%
OTU_230	26	11	KJ921524	AJ563890	Glomeraceae	Glomus	VTX00137	432	e-122	225/225	100.00%
OTU_466	25	24	KJ921573	AJ854081	Glomeraceae	Glomus	VTX00216	406	e-114	220/225	97.80%
OTU_286	24	7	KJ921534	AM849307	Diversisporaceae	Diversispora	VTX00054	432	e-122	225/225	100.00%
OTU_318	24	10	KJ921546	HE799069	Glomeraceae	Glomus	VTX00214	408	e-115	213/225	94.70%
OTU_225	23	1	KJ921523	HE799010	Glomeraceae	Glomus	VTX00166	432	e-122	225/225	100.00%
OTU_471	23	9	KJ921577	HF567017	Glomeraceae	Glomus	VTX00115	414	e-116	221/225	98.20%
OTU_385	22	12	KJ921562	HF567017	Glomeraceae	Glomus	VTX00115	412	e-116	211/225	93.80%
OTU_399	22	7	KP064040	HQ323546	Glomeraceae	Glomus	VTX00143	396	e-111	220/225	97.70%
OTU_418	22	9	KJ921567	HE799083	Glomeraceae	Glomus	VTX00219	400	e-112	221/225	98.20%
OTU_285	21	9	KJ921533	AB193051	Claroideoglomeraceae	Claroideoglomus	VTX00279	369	e-103	216/225	96.00%
OTU_491	21	5	KJ921586	AJ309465	Glomeraceae	Glomus	VTX00143	406	e-114	212/225	94.20%
OTU_304	19	8	KJ921542	HE659888	Glomeraceae	Glomus	VTX00064	143	e-35	214/225	95.10%
OTU_503	19	11	KJ921589	AB193051	Claroideoglomeraceae	Claroideoglomus	VTX00279	363	e-101	212/225	94.20%
OTU_219	17	5	KJ921520	GU353940	Glomeraceae	Glomus	VTX00086	424	e-119	224/225	99.60%
OTU_283	15	10	KJ921532	JN009476	Diversisporaceae	Diversispora	VTX00062	424	e-119	224/225	99.60%
OTU_380	15	3	KJ921559	FJ875130	Glomeraceae	Glomus	VTX00163	396	e-111	219/225	97.30%
OTU_291	14	5	KJ921536	FR848023	Glomeraceae	Glomus	VTX00160	369	e-103	217/225	96.40%
OTU_305	13	2	KP064041	FM877526	Glomeraceae	Glomus	VTX00105	432	e-122	225/225	100.00%
OTU_280	12	4	KJ921531	FR728622	Gigasporaceae	Scutellospora	VTX00052	440	e-124	222/225	98.70%
OTU_407	12	5	KJ921565	FR848078	Claroideoglomeraceae	Claroideoglomus	VTX00225	361	e-100	216/225	96.00%
OTU_296	11	5	KJ921537	JN009472	Glomeraceae	Glomus	VTX00137	426	e-120	222/225	98.70%
OTU_357	11	3	KJ921554	FJ875130	Glomeraceae	Glomus	VTX00163	396	e-111	221/225	98.20%
OTU_300	7	3	KJ921539	HF567267	Glomeraceae	Glomus	VTX00222	383	e-107	217/225	96.40%
OTU_484	7	3	KJ921584	HE798982	Glomeraceae	Glomus	VTX00163	385	e-107	219/225	97.30%
OTU_314	6	1	KJ921545	HF566925	Glomeraceae	Glomus	VTX00108	446	e-126	225/225	100.00%
OTU_442	5	2	KJ921571	HE799268	Claroideoglomeraceae	Claroideoglomus	VTX00057	385	e-107	211/225	93.80%
OTU_467	5	1	KJ921574	AB193051	Claroideoglomeraceae	Claroideoglomus	VTX00279	335	e-92	213/225	94.70%
OTU_443	4	1	KJ921572	HE615073	Claroideoglomeraceae	Claroideoglomus	VTX00402	379	e-106	220/225	97.80%
OTU_445	2	1	KP064042	HE799164	Glomeraceae	Glomus	VTX00072	404	e-113	222/225	98.60%

Appendix C

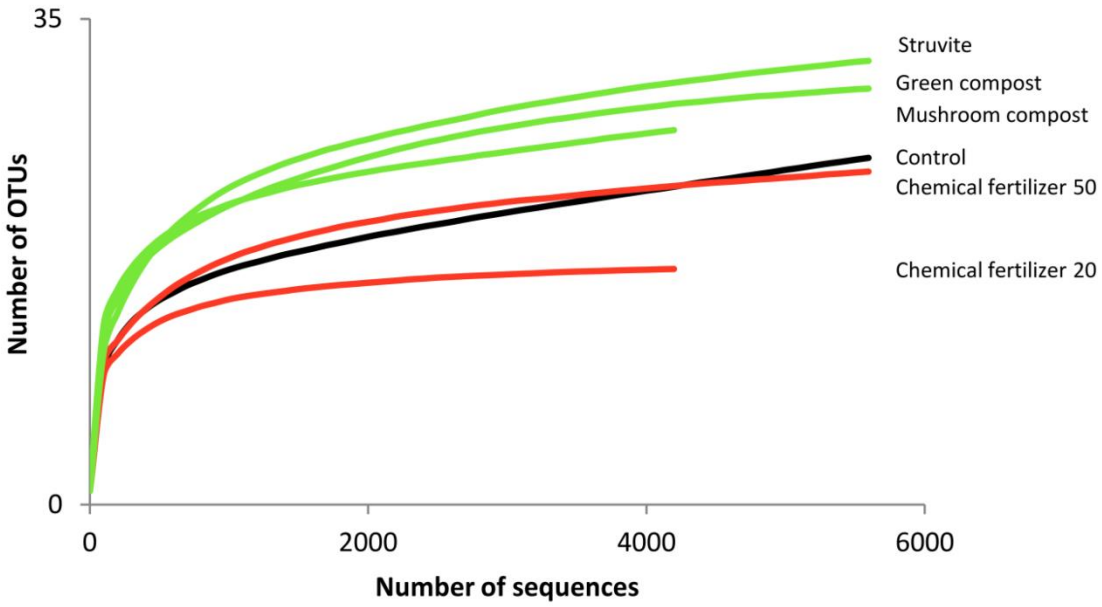


Figure C1 Rarefaction curves of arbuscular mycorrhizal fungal operational taxonomic units for all treatments. The chemical and slow-release fertilizers are shown in red and green, respectively. Each treatment consisted of four samples (1399 sequences per sample), except the Chemical fertilizer 20 and the Mushroom compost treatment which consisted of only three samples after rarefying. All rarefaction curves tended to saturation.

Table C1 List of the 39 operational taxonomic units (OTUs), identified at a 3% sequence dissimilarity cut-off discovered in chapter 4. The taxonomic affiliations were obtained by BLAST analysis against the MaarjAM database.

OTU ID	Number of reads (rarefied dataset)	Family	Genus	Virtual Taxa	Closest match MaarjAM ID	Bitscore	e-value	Identity (bp)	Identity (%)
OTU_02	8990	Glomeraceae	Glomus	VTX00113	HE659733	454	e-128	232/233	99.57%
OTU_01	8181	Claroideoglomeraceae	Claroideoglomus	VTX00057	HE799266	462	e-130	233/233	100.00%
OTU_03	2772	Claroideoglomeraceae	Claroideoglomus	VTX00225	HQ993241	462	e-130	233/233	100.00%
OTU_54	2665	Glomeraceae	Glomus	VTX00115	AB749502	430	e-121	229/233	98.28%
OTU_04	1986	Glomeraceae	Glomus	VTX00163	HF566628	448	e-126	233/233	100.00%
OTU_07	1918	Glomeraceae	Glomus	VTX00163	FJ875130	454	e-128	232/233	99.57%
OTU_05	1062	Glomeraceae	Glomus	VTX00160	FR848023	462	e-130	233/233	100.00%
OTU_06	811	Glomeraceae	Glomus	VTX00214	HM215868	462	e-130	233/233	100.00%
OTU_50	567	Glomeraceae	Glomus	VTX00342	FM876883	430	e-121	230/233	98.71%
OTU_14	235	Glomeraceae	Glomus	VTX00156	JX999426	462	e-130	233/233	100.00%
OTU_16	174	Glomeraceae	Glomus	VTX00064	HE659885	454	e-128	232/233	99.57%
OTU_17	174	Glomeraceae	Glomus	VTX00130	AB749467	454	e-128	232/233	99.57%
OTU_13	169	Glomeraceae	Glomus	VTX00159	AY499494	446	e-126	231/233	99.14%
OTU_27	129	Glomeraceae	Glomus	VTX00115	JN009217	416	e-117	229/233	98.28%
OTU_67	129	Claroideoglomeraceae	Claroideoglomus	VTX00225	FR848078	440	e-124	232/233	99.57%
OTU_32	122	Glomeraceae	Glomus	VTX00153	AF437659	448	e-126	233/233	100.00%
OTU_19	117	Glomeraceae	Glomus	VTX00163	HE798986	462	e-130	233/233	100.00%
OTU_74	114	Glomeraceae	Glomus	VTX00113	HF566562	444	e-125	230/233	98.71%
OTU_18	87	Claroideoglomeraceae	Claroideoglomus	VTX00193	KF049810	462	e-130	233/233	100.00%
OTU_21	67	Glomeraceae	Glomus	VTX00186	HE799032	432	e-122	231/233	99.14%
OTU_33	55	Glomeraceae	Glomus	VTX00064	EU340314	454	e-128	232/233	99.57%
OTU_81	53	Glomeraceae	Glomus	VTX00214	HE799069	430	e-121	230/233	98.71%
OTU_22	36	Glomeraceae	Glomus	VTX00199	KC708342	462	e-130	233/233	100.00%
OTU_42	22	Claroideoglomeraceae	Claroideoglomus	VTX00340	FN869794	432	e-122	231/233	99.14%
OTU_23	21	Glomeraceae	Glomus	VTX00155	HE613474	448	e-126	233/233	100.00%
OTU_25	16	Claroideoglomeraceae	Claroideoglomus	VTX00225	KF386299	424	e-119	230/233	98.71%
OTU_38	15	Glomeraceae	Glomus	VTX00125	HQ342704	448	e-126	233/233	100.00%
OTU_93	15	Glomeraceae	Glomus	VTX00153	DQ085239	432	e-122	231/233	99.14%
OTU_43	14	Glomeraceae	Glomus	VTX00222	JN559801	440	e-124	232/233	99.57%
OTU_45	13	Glomeraceae	Glomus	VTX00151	GU353928	462	e-130	233/233	100.00%
OTU_58	11	Glomeraceae	Glomus	VTX00222	AB076272	462	e-130	233/233	100.00%
OTU_69	6	Claroideoglomeraceae	Claroideoglomus	VTX00279	AB193051	302	e-105	226/233	97.00%
OTU_71	6	Claroideoglomeraceae	Claroideoglomus	VTX00056	JN009183	454	e-128	232/233	99.57%
OTU_73	6	Paraglomeraceae	Paraglomus	VTX00281	HE613491	462	e-130	233/233	100.00%
OTU_28	5	Claroideoglomeraceae	Claroideoglomus	VTX00055	JN252441	448	e-126	233/233	100.00%
OTU_72	5	Claroideoglomeraceae	Claroideoglomus	VTX00225	HQ323487	408	e-114	228/233	97.85%
OTU_47	4	Glomeraceae	Glomus	VTX00222	JQ654576	448	e-126	233/233	100.00%
OTU_56	3	Glomeraceae	Glomus	VTX00129	EF409064	462	e-130	233/233	100.00%
OTU_75	1	Glomeraceae	Glomus	VTX00222	JN009276	414	e-116	227/233	97.42%

Appendix D

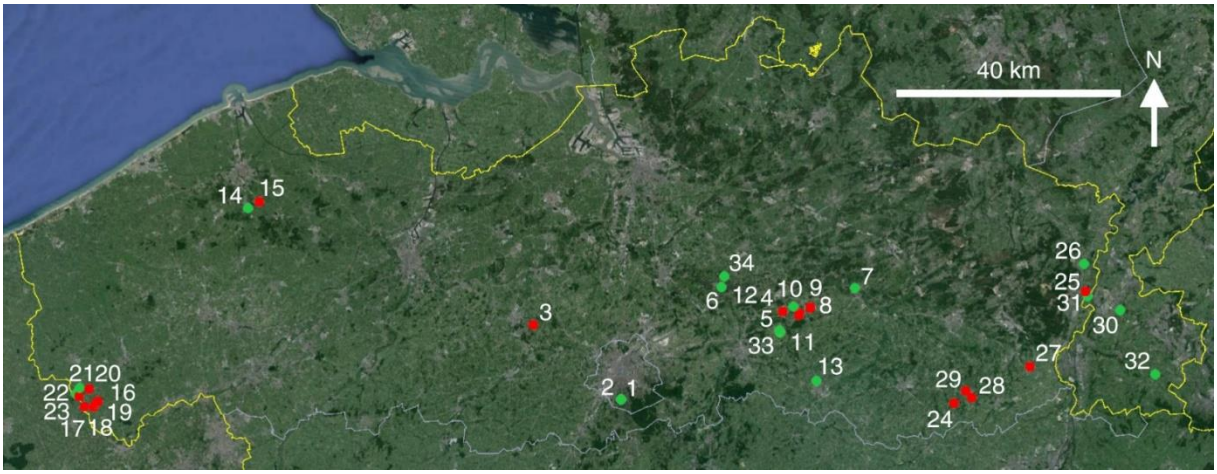
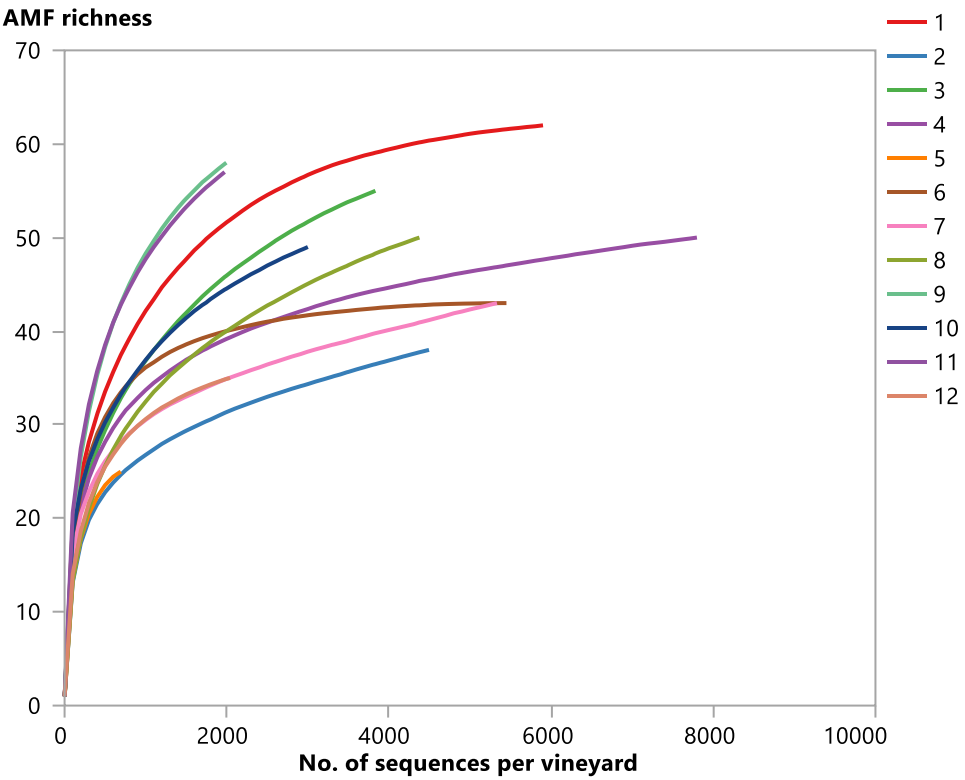
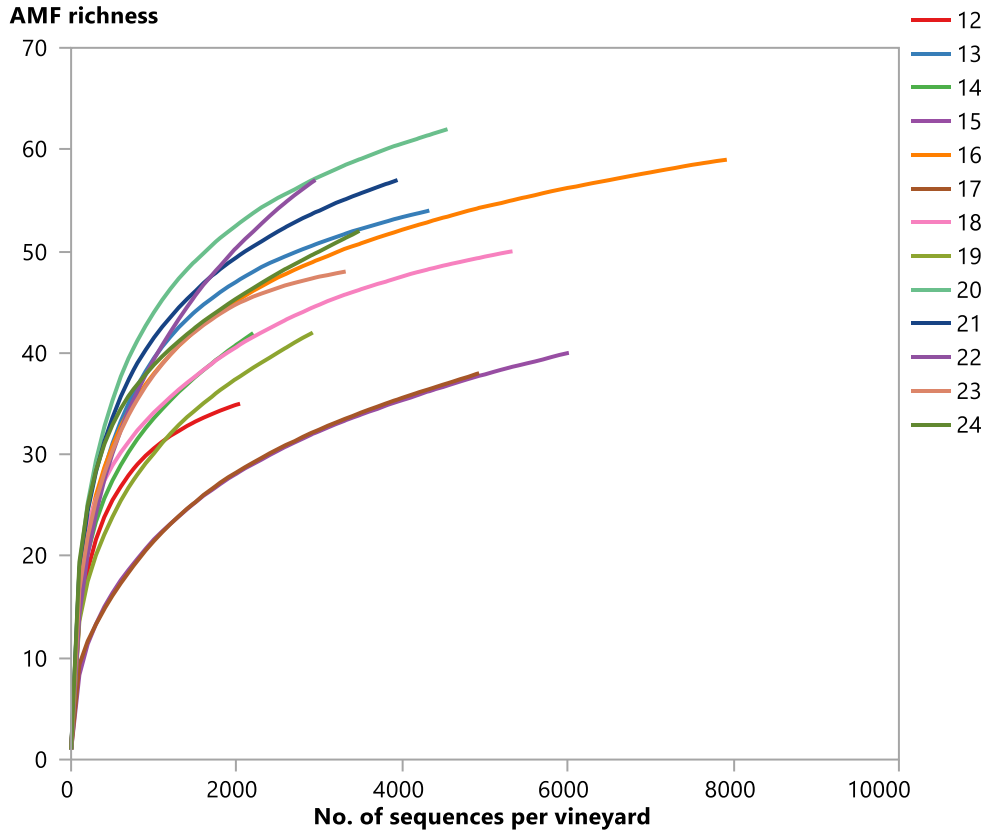


Figure D1 Map of Flanders (Belgium) showing the distribution of the 34 sampled vineyards with organic (green) and conventional (red) management.

(a)


AMF richness
70
60
50
40
30
20
10
0
0
2000
4000
6000
8000
10000
No. of sequences per vineyard
1
2
3
4
5
6
7
8
9
10
11
12

(b)


AMF richness
70
60
50
40
30
20
10
0
0
2000
4000
6000
8000
10000
No. of sequences per vineyard
12
13
14
15
16
17
18
19
20
21
22
23
24

(c)

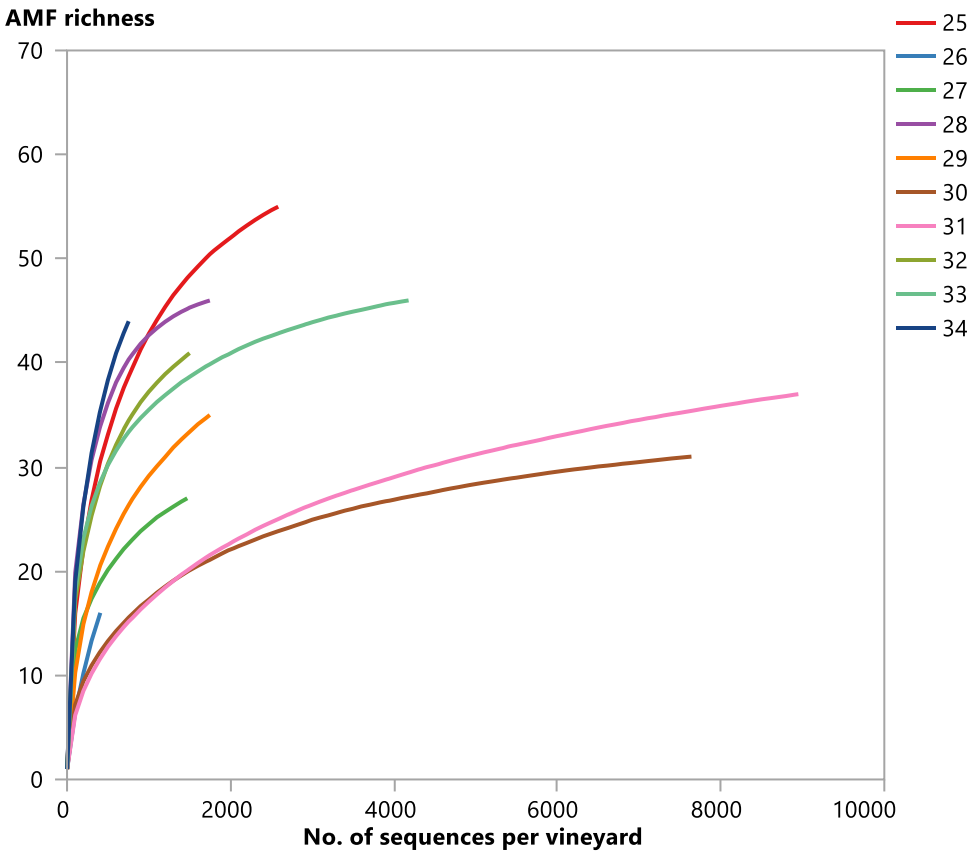


Figure D2 Rarefaction curves of AMF richness for all 34 vineyards. For the sake of graphical representation, the curves are shown in three separate graphs, (a), (b) and (c). Vineyards are shown in different colors.

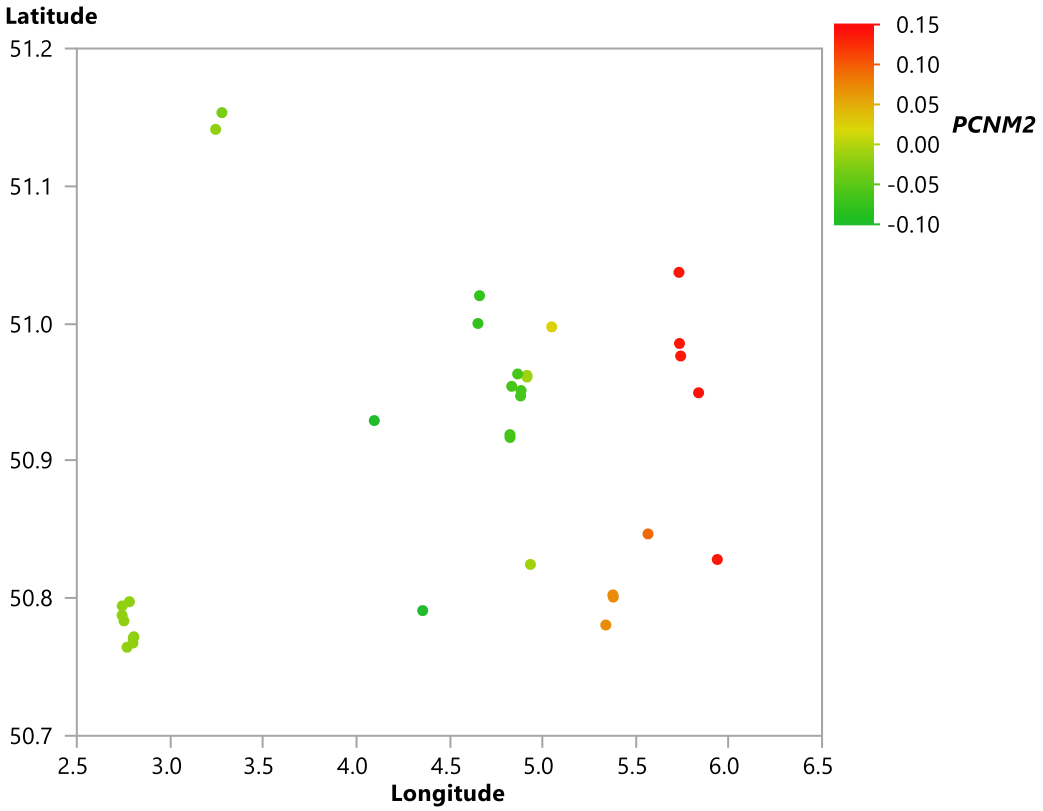


Figure D3 The relationship between geographical location (latitude and longitude) and the spatial predictor PCNM2 that significantly contributed to AMF community composition. PCNM2 separates the sampled vineyards according to their longitude and increases with higher longitudes.

Table D1 Coordinates, soil properties, management and AMF diversity measures of all 34 vineyards sampled in chapter 5.

VineyardID	Latitude	Longitude	Management	No. of years organic management	Age (yr)	pH*	Soil N (mg/kg)*	Olsen P (mg/kg)*	Organic carbon (%)*	Cu (mg/kg)*	AMF richness*	Exp(Shannon)*
1	50.791083	4.356369	Organic	5	5	7.0 (0.19)	13.30 (1.06)	10.42 (1.66)	3.85% (0.16)	15.9 (1.1)	24.40 (5.73)	5.55 (1.28)
2	50.790937	4.356087	Organic	15	15	7.5 (0.10)	17.45 (1.60)	14.25 (3.22)	4.80% (0.45)	18.9 (2.1)	13.80 (3.02)	4.17 (0.62)
3	50.929328	4.096563	Conventional		18	7.2 (0.06)	10.64 (1.05)	37.57 (2.21)	3.28% (0.16)	34.2 (1.3)	23.40 (1.75)	4.61 (1.25)
4	50.95423	4.834063	Conventional		14	8.0 (0.14)	11.33 (1.57)	19.00 (7.37)	5.62% (0.78)	14.7 (4.4)	18.80 (3.73)	4.81 (0.78)
5	50.947195	4.881944	Conventional		20	7.2 (0.20)	21.19 (6.84)	89.52 (7.21)	3.61% (0.27)	30.8 (3.6)	7.60 (2.42)	3.18 (0.66)
6	50.999955	4.653144	Organic	3.5	3.5	7.4 (0.25)	6.19 (0.58)	12.17 (1.14)	2.68% (0.45)	2.0 (0.2)	19.80 (3.87)	7.96 (1.82)
7	50.997462	5.048845	Organic	5	10	7.3 (0.28)	8.08 (0.73)	35.47 (0.61)	5.50% (0.36)	21.8 (0.7)	20.60 (1.94)	6.17 (1.32)
8	50.962182	4.916307	Conventional		3	6.8 (0.19)	12.98 (1.02)	21.75 (4.27)	4.32% (0.15)	5.3 (0.4)	15.80 (3.48)	4.01 (1.50)
9	50.960943	4.916032	Conventional		12	7.5 (0.23)	11.31 (2.18)	13.71 (4.16)	3.21% (0.24)	13.4 (3.9)	24.20 (3.90)	6.22 (0.85)
10	50.963228	4.866117	Organic	6	6	7.2 (0.21)	6.58 (1.26)	26.47 (2.01)	3.36% (0.14)	7.0 (0.3)	19.40 (3.80)	7.59 (1.15)
11	50.917011	4.825176	Organic	9	9	7.3 (0.09)	23.70 (3.34)	37.19 (2.54)	13.91% (7.51)	16.6 (1.8)	26.60 (2.79)	10.53 (2.11)
12	50.951173	4.884415	Conventional		12	6.9 (0.27)	9.81 (1.54)	75.12 (8.34)	4.80% (0.45)	5.8 (0.6)	11.40 (1.54)	4.49 (1.30)
13	50.824648	4.934691	Organic	18	18	7.1 (0.07)	13.53 (3.26)	57.20 (2.44)	4.27% (0.28)	41.4 (3.3)	21.40 (3.43)	4.58 (1.23)
14	51.141171	3.245037	Organic	3	4	6.9 (0.05)	7.62 (1.84)	77.95 (4.23)	4.12% (0.25)	11.4 (0.6)	13.40 (3.34)	3.58 (0.90)
15	51.153338	3.278780	Conventional		5	7.4 (0.07)	14.38 (1.23)	77.40 (8.38)	5.33% (0.65)	15.2 (1.5)	15.80 (3.01)	2.89 (0.47)
16	50.771107	2.802965	Conventional		16	7.4 (0.10)	13.22 (1.01)	59.97 (3.74)	3.93% (0.18)	41.7 (2.8)	22.60 (6.73)	4.62 (1.40)
17	50.764404	2.769497	Conventional		6	7.5 (0.11)	25.65 (2.66)	29.29 (6.68)	6.11% (0.50)	25.2 (1.7)	16.80 (2.78)	4.40 (0.60)
18	50.767484	2.801193	Conventional		19	6.9 (0.25)	18.63 (4.59)	26.60 (11.32)	4.92% (0.22)	59.5 (2.6)	16.80 (4.45)	4.66 (1.41)
19	50.772038	2.806249	Conventional		11	7.1 (0.05)	15.45 (0.74)	32.01 (1.69)	3.81% (0.30)	29.7 (0.8)	20.60 (1.50)	4.22 (0.58)
20	50.797547	2.782298	Conventional		4	7.1 (0.17)	14.75 (0.58)	45.91 (4.92)	3.36% (0.17)	21.7 (1.4)	22.60 (5.15)	5.32 (1.67)
21	50.794472	2.743879	Organic	1	5	7.8 (0.12)	17.25 (0.54)	65.94 (20.71)	3.90% (0.29)	20.6 (1.3)	22.20 (3.64)	6.68 (1.03)
22	50.787666	2.742963	Organic	15	15	7.4 (0.08)	16.32 (2.32)	45.71 (2.32)	4.05% (0.22)	42.5 (7.8)	17.80 (4.28)	4.82 (1.10)
23	50.783575	2.753057	Conventional		10	7.0 (0.20)	15.89 (0.95)	44.06 (2.53)	3.55% (0.15)	24.4 (1.8)	21.80 (4.14)	6.89 (1.11)
24	50.780609	5.339358	Conventional		5	7.5 (0.13)	22.65 (0.68)	73.46 (10.10)	4.19% (0.11)	11.7 (2.6)	20.60 (4.21)	5.59 (0.67)
25	50.985410	5.734681	Conventional		1	7.6 (0.13)	11.39 (0.88)	38.03 (2.26)	4.85% (0.40)	20.2 (2.1)	21.80 (3.72)	7.07 (1.86)
26	51.037157	5.732011	Organic	1	2.5	7.1 (0.12)	17.90 (1.11)	128.36 (6.76)	6.96% (0.30)	28.0 (1.8)	5.20 (0.37)	1.91 (0.08)
27	50.846857	5.565825	Conventional		3	7.3 (0.29)	11.71 (0.44)	59.26 (13.97)	4.66% (0.23)	19.2 (1.8)	10.80 (2.50)	3.82 (0.65)
28	50.800899	5.379411	Conventional		15	7.5 (0.16)	14.53 (0.71)	60.16 (4.86)	4.10% (0.13)	22.4 (1.2)	20.80 (2.06)	5.96 (1.58)
29	50.802502	5.377195	Conventional		10	7.2 (0.27)	8.59 (1.71)	82.35 (2.10)	3.16% (0.25)	11.3 (1.1)	11.60 (4.87)	5.22 (3.23)
30	50.949533	5.837307	Organic	5	5	7.4 (0.12)	15.47 (1.05)	52.72 (5.47)	4.45% (0.30)	18.0 (0.3)	12.00 (1.92)	3.27 (0.77)
31	50.976294	5.741070	Organic	2	2	7.6 (0.12)	19.96 (0.67)	50.22 (5.04)	6.44% (0.36)	28.0 (1.1)	11.60 (3.72)	2.07 (0.42)
32	50.828264	5.937815	Organic	15	15	7.5 (0.21)	39.17 (2.06)	20.97 (3.81)	12.47% (0.57)	16.9 (0.6)	18.20 (2.52)	6.73 (0.75)
33	50.919112	4.824676	Organic	9	9	7.5 (0.09)	16.81 (0.75)	51.88 (3.34)	5.90% (0.34)	30.8 (1.9)	20.00 (3.07)	7.44 (1.71)
34	51.020183	4.661539	Organic	6	6	7.0 (0.17)	29.80 (5.47)	102.37 (4.68)	4.92% (0.23)	6.7 (1.4)	14.80 (1.71)	5.01 (0.25)

*S.E. between brackets

Table D2 List of the 123 operational taxonomic units (OTUs), identified at a 3% sequence dissimilarity cut-off discovered in chapter 5. The taxonomic affiliations were obtained by BLAST analysis against the MaarjAM database.

OTU ID	Number of reads	Closest match Maarjam ID	Family	Genus	MaarjAM Virtual Taxa ID	MaarjAM Bitscore	MaarjAM e-value	Identity (bp)	Identity (%)
OTU_2	58385	HF677909	Glomeraceae	Glomus	VTX00113	446	e-125	225/225	100.0%
OTU_13	8261	HF678085	Glomeraceae	Glomus	VTX00214	446	e-125	225/225	100.0%
OTU_1002	7401	LN622548	Glomeraceae	Glomus	VTX00114	430	e-121	224/225	99.6%
OTU_33	3548	AB195628	Claroideoglomeraceae	Claroideoglomus	VTX00278	394	e-110	218/225	97.2%
OTU_17	3498	HF678074	Glomeraceae	Glomus	VTX00057	446	e-125	225/225	100.0%
OTU_25	2885	LN620617	Glomeraceae	Glomus	VTX00153	446	e-125	225/225	100.0%
OTU_34	2706	LN621289	Glomeraceae	Glomus	VTX00151	438	e-123	224/225	99.6%
OTU_27	2282	JN090200	Glomeraceae	Glomus	VTX00069	432	e-121	225/225	100.0%
OTU_30	2079	JN009369	Glomeraceae	Glomus	VTX00064	432	e-121	225/225	100.0%
OTU_35	1988	HF568348	Glomeraceae	Glomus	VTX00135	446	e-125	225/225	100.0%
OTU_49	1938	LN622034	Glomeraceae	Glomus	VTX00219	432	e-121	225/225	100.0%
OTU_59	1861	FR848023	Glomeraceae	Glomus	VTX00160	432	e-121	225/225	100.0%
OTU_1782	1752	HF566988	Glomeraceae	Glomus	VTX00114	398	e-111	219/225	97.3%
OTU_45	1581	HF677967	Glomeraceae	Glomus	VTX00212	446	e-125	225/225	100.0%
OTU_32	1370	HE799164	Glomeraceae	Glomus	VTX00072	430	e-121	223/225	99.1%
OTU_79	1247	LN622983	Glomeraceae	Glomus	VTX00155	446	e-125	225/225	100.0%
OTU_82	1222	LN621845	Glomeraceae	Glomus	VTX00199	446	e-125	225/225	100.0%
OTU_1177	1202	HF954811	Glomeraceae	Glomus	VTX00326	438	e-123	224/225	99.6%
OTU_142	1176	AJ309465	Glomeraceae	Glomus	VTX00143	432	e-121	225/225	100.0%
OTU_141	1162	HQ323487	Claroideoglomeraceae	Claroideoglomus	VTX00225	432	e-121	225/225	100.0%
OTU_65	1148	EF041081	Claroideoglomeraceae	Claroideoglomus	VTX00225	432	e-121	225/225	100.0%
OTU_98	1031	GU353940	Glomeraceae	Glomus	VTX00086	424	e-119	224/225	99.6%
OTU_134	1029	FR728572	Glomeraceae	Glomus	VTX00214	446	e-125	225/225	100.0%
OTU_851	953	AB195628	Claroideoglomeraceae	Claroideoglomus	VTX00278	377	e-105	218/225	97.2%
OTU_87	923	HF954611	Glomeraceae	Glomus	VTX00064	428	e-120	225/225	100.0%
OTU_1465	699	JN090188	Glomeraceae	Glomus	VTX00143	414	e-116	222/225	98.7%
OTU_653	694	AB195628	Claroideoglomeraceae	Claroideoglomus	VTX00278	381	e-106	218/225	97.2%
OTU_60	673	LN619570	Glomeraceae	Glomus	VTX00069	416	e-117	223/225	99.1%
OTU_130	631	HF568338	Glomeraceae	Glomus	VTX00125	432	e-121	225/225	100.0%
OTU_1951	617	LN621304	Glomeraceae	Glomus	VTX00064	422	e-118	222/225	98.7%
OTU_409	613	LN620469	Glomeraceae	Glomus	VTX00153	446	e-125	225/225	100.0%
OTU_125	605	LN621703	Glomeraceae	Glomus	VTX00143	446	e-125	225/225	100.0%
OTU_104	601	LN621736	Glomeraceae	Glomus	VTX00163	446	e-125	225/225	100.0%
OTU_123	581	HF677983	Glomeraceae	Glomus	VTX00193	438	e-123	224/225	99.6%
OTU_150	575	GU353680	Glomeraceae	Glomus	VTX00309	432	e-121	225/225	100.0%
OTU_93	542	LN619908	Claroideoglomeraceae	Claroideoglomus	VTX00056	446	e-125	225/225	100.0%
OTU_185	515	LN622846	Glomeraceae	Glomus	VTX00172	430	e-121	223/225	99.1%
OTU_77	501	LN623107	Glomeraceae	Glomus	VTX00074	446	e-125	225/225	100.0%
OTU_149	500	FN869794	Claroideoglomeraceae	Claroideoglomus	VTX00340	432	e-121	225/225	100.0%
OTU_357	499	FJ867636	Glomeraceae	Glomus	VTX00113	406	e-114	220/225	97.8%
OTU_171	459	KF386336	Glomeraceae	Glomus	VTX00309	432	e-121	225/225	100.0%
OTU_235	441	HF568555	Glomeraceae	Glomus	VTX00222	446	e-125	225/225	100.0%
OTU_267	419	LN623127	Glomeraceae	Glomus	VTX00163	446	e-125	225/225	100.0%
OTU_1129	390	LN621716	Glomeraceae	Glomus	VTX00214	406	e-114	220/225	97.8%
OTU_1673	352	LN621716	Glomeraceae	Glomus	VTX00214	400	e-112	221/225	98.2%
OTU_128	332	HF677952	Glomeraceae	Glomus	VTX00122	446	e-125	225/225	100.0%
OTU_1653	299	LN622950	Glomeraceae	Glomus	VTX00214	398	e-111	219/225	97.3%
OTU_1384	295	AF437659	Glomeraceae	Glomus	VTX00153	422	e-118	222/225	98.7%
OTU_157	272	HF568615	Paraglomeraceae	Paraglomus	VTX00281	446	e-125	225/225	100.0%
OTU_1417	241	LN620430	Claroideoglomeraceae	Claroideoglomus	VTX00056	424	e-119	224/225	99.6%
OTU_196	237	HQ342701	Gigasporaceae	Scutellospora	VTX00052	432	e-121	225/225	100.0%
OTU_724	236	JN090188	Glomeraceae	Glomus	VTX00143	422	e-118	222/225	98.7%
OTU_942	233	AJ301857	Glomeraceae	Glomus	VTX00199	438	e-123	224/225	99.6%
OTU_1500	199	HQ342721	Glomeraceae	Glomus	VTX00155	422	e-118	222/225	98.7%
OTU_326	196	LN622494	Glomeraceae	Glomus	VTX00153	424	e-119	224/225	99.6%
OTU_495	188	LN620118	Glomeraceae	Glomus	VTX00149	446	e-125	225/225	100.0%
OTU_1340	182	HF954647	Glomeraceae	Glomus	VTX00222	420	e-118	224/225	99.6%
OTU_1753	179	LN620616	Glomeraceae	Glomus	VTX00143	438	e-123	224/225	99.6%
OTU_263	170	HF678017	Claroideoglomeraceae	Claroideoglomus	VTX00222	432	e-121	225/225	100.0%
OTU_222	156	AB193051	Claroideoglomeraceae	Claroideoglomus	VTX00279	357	e-99	216/225	97.1%
OTU_211	137	KF386299	Claroideoglomeraceae	Claroideoglomus	VTX00225	424	e-119	224/225	99.6%
OTU_789	135	LN621794	Glomeraceae	Glomus	VTX00342	444	e-125	224/225	99.6%
OTU_1835	131	AJ563867	Glomeraceae	Glomus	VTX00130	424	e-119	224/225	99.6%

OTU_262	114	HF568238	Glomeraceae	Glomus	VTX00067	446	e-125	225/225	100.0%
OTU_315	107	LN616178	Glomeraceae	Glomus	VTX00153	373	e-103	218/225	97.2%
OTU_1438	105	HF678013	Glomeraceae	Glomus	VTX00219	398	e-111	220/225	97.8%
OTU_1481	102	FR728594	Glomeraceae	Glomus	VTX00219	408	e-114	219/225	97.3%
OTU_577	96	FJ194508	Glomeraceae	Glomus	VTX00223	422	e-118	222/225	98.7%
OTU_664	92	HE775380	Glomeraceae	Glomus	VTX00191	446	e-125	225/225	100.0%
OTU_478	89	LN622828	Glomeraceae	Glomus	VTX00122	438	e-123	224/225	99.6%
OTU_582	89	LN623479	Glomeraceae	Glomus	VTX00129	446	e-125	225/225	100.0%
OTU_432	86	HF954875	Glomeraceae	Glomus	VTX00222	446	e-125	225/225	100.0%
OTU_374	84	LN619570	Glomeraceae	Glomus	VTX00069	381	e-106	219/225	97.3%
OTU_554	75	HF677933	Glomeraceae	Glomus	VTX00108	446	e-125	225/225	100.0%
OTU_363	72	HF568239	Glomeraceae	Glomus	VTX00069	446	e-125	225/225	100.0%
OTU_397	72	LN620186	Diversisporaceae	Diversispora	VTX00060	446	e-125	225/225	100.0%
OTU_1901	71	FN556647	Glomeraceae	Glomus	VTX00163	400	e-112	221/225	98.2%
OTU_459	70	HM215643	Claroideoglomeraceae	Claroideoglomus	VTX00225	408	e-114	222/225	98.7%
OTU_1618	67	LN615743	Glomeraceae	Glomus	VTX00222	438	e-123	224/225	99.6%
OTU_649	64	HF954853	Glomeraceae	Glomus	VTX00065	446	e-125	225/225	100.0%
OTU_332	60	GU598368	Glomeraceae	Glomus	VTX00130	438	e-123	224/225	99.6%
OTU_1536	59	LN622889	Glomeraceae	Glomus	VTX00115	432	e-121	221/225	98.2%
OTU_411	58	JN252444	Gigasporaceae	Scutellospora	VTX00041	438	e-123	224/225	99.6%
OTU_910	56	LN618845	Diversisporaceae	Diversispora	VTX00061	432	e-121	225/225	100.0%
OTU_1134	50	LN623093	Glomeraceae	Glomus	VTX00222	408	e-114	222/225	98.7%
OTU_574	50	GU353728	Gigasporaceae	Scutellospora	VTX00049	432	e-121	225/225	100.0%
OTU_558	46	LN621738	Glomeraceae	Glomus	VTX00186	430	e-121	223/225	99.1%
OTU_878	46	HF954714	Claroideoglomeraceae	Claroideoglomus	VTX00056	432	e-121	225/225	100.0%
OTU_1878	44	LN621679	Glomeraceae	Glomus	VTX00199	410	e-115	219/225	97.3%
OTU_424	31	LN622564	Glomeraceae	Glomus	VTX00172	432	e-121	225/225	100.0%
OTU_1551	27	LN621967	Glomeraceae	Glomus	VTX00222	406	e-114	220/225	97.8%
OTU_1191	25	AJ852605	Gigasporaceae	Gigaspora	VTX00039	446	e-125	225/225	100.0%
OTU_1144	24	AJ854088	Glomeraceae	Glomus	VTX00143	400	e-112	221/225	98.2%
OTU_1512	24	LN623042	Glomeraceae	Glomus	VTX00163	414	e-116	222/225	98.7%
OTU_890	24	HE615073	Claroideoglomeraceae	Claroideoglomus	VTX00402	385	e-107	219/225	97.3%
OTU_1066	22	LN621534	Glomeraceae	Glomus	VTX00115	408	e-114	218/225	97.2%
OTU_1810	21	EU123443	Glomeraceae	Glomus	VTX00143	392	e-109	220/225	97.8%
OTU_754	21	GU353726	Gigasporaceae	Scutellospora	VTX00052	432	e-121	225/225	100.0%
OTU_823	21	HF568281	Glomeraceae	Glomus	VTX00105	446	e-125	225/225	100.0%
OTU_687	19	KF386269	Paraglomeraceae	Paraglomus	VTX00335	416	e-117	223/225	99.1%
OTU_708	18	LN621595	Glomeraceae	Glomus	VTX00166	398	e-111	220/225	97.8%
OTU_666	17	LN623119	Glomeraceae	Glomus	VTX00130	414	e-116	221/225	98.2%
OTU_1316	15	LN616433	Acaulosporaceae	Acaulospora	VTX00030	432	e-121	225/225	100.0%
OTU_836	15	AY129571	Glomeraceae	Glomus	VTX00200	424	e-119	224/225	99.6%
OTU_886	15	HF677831	Diversisporaceae	Diversispora	VTX00074	402	e-112	216/225	97.1%
OTU_971	15	AB076344	Claroideoglomeraceae	Claroideoglomus	VTX00278	432	e-121	225/225	100.0%
OTU_992	15	HF954901	Glomeraceae	Glomus	VTX00137	430	e-121	224/225	99.6%
OTU_1076	13	HG004533	Claroideoglomeraceae	Claroideoglomus	VTX00193	424	e-119	224/225	99.6%
OTU_1487	13	HF954873	Claroideoglomeraceae	Claroideoglomus	VTX00193	446	e-125	225/225	100.0%
OTU_1193	12	AY499494	Glomeraceae	Glomus	VTX00159	430	e-121	223/225	99.1%
OTU_1818	12	LN616476	Glomeraceae	Glomus	VTX00088	446	e-125	225/225	100.0%
OTU_1146	11	LN616419	Glomeraceae	Glomus	VTX00191	432	e-121	225/225	100.0%
OTU_1600	11	HF678030	Glomeraceae	Glomus	VTX00061	416	e-117	219/225	97.3%
OTU_1137	10	LN617068	Gigasporaceae	Scutellospora	VTX00052	385	e-107	219/225	97.3%
OTU_1548	8	HF954588	Glomeraceae	Glomus	VTX00186	392	e-109	220/225	97.8%
OTU_1212	7	LN620023	Archaeosporaceae	Archaeospora	VTX00006	438	e-123	224/225	99.6%
OTU_1445	6	HE576932	Claroideoglomeraceae	Claroideoglomus	VTX00357	327	e-90	218/225	97.2%
OTU_1889	6	HF677990	Glomeraceae	Glomus	VTX00115	422	e-118	222/225	98.7%
OTU_1900	6	LN621700	Glomeraceae	Glomus	VTX00129	438	e-123	224/225	99.6%
OTU_1937	6	LN616154	Glomeraceae	Glomus	VTX00233	446	e-125	225/225	100.0%
OTU_1071	5	LN621030	Claroideoglomeraceae	Claroideoglomus	VTX00193	414	e-116	221/225	98.2%
OTU_1140	5	EU340326	Gigasporaceae	Gigaspora	VTX00039	375	e-104	216/225	97.1%
OTU_1898	5	LN621045	Acaulosporaceae	Acaulospora	VTX00015	446	e-125	225/225	100.0%

Appendix E

Appendix E1 References to studies used in the meta-analysis.

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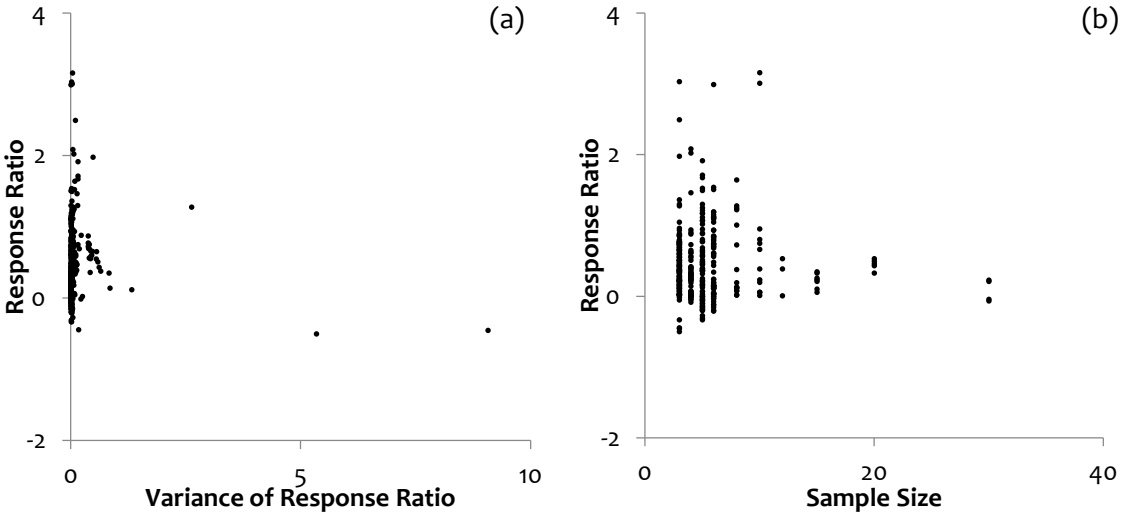
Appendix E2 Scatter plots of effect sizes of total biomass (a & b) versus their variance and the sample size of each experiment did not show any patterns indicative of publication bias, for example no relation between effect size and sample size could be discovered.



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